For Reference

NOT TO BE TAKEN FROM THIS ROOM

For Reference

NOT TO BE TAKEN FROM THIS ROOM

Ex LIBRIS
UNIVERSITATIS
ALBERTAENSIS

University of Alberta
Printing Department

Digitized by the Internet Archive
in 2019 with funding from
University of Alberta Libraries

https://archive.org/details/Boudreau1964

964

THE UNIVERSITY OF ALBERTA

THE SPECIFIC DISTRIBUTION OF FATTY ACIDS IN THE GLYCERIDES OF MILK FAT

by

ARMAND BOUDREAU, L.S.A.

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF DAIRY SCIENCE

EDMONTON ALBERTA

APRIL, 1964

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled

THE SPECIFIC DISTRIBUTION OF FATTY ACIDS IN THE GLYCERIDES OF MILK FAT

submitted by A. Boudreau in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ACKNOWLEDGEMENTS

The writer is indebted to the staff of the Department of Dairy Science and particularly desires to acknowledge the guidance and constructive criticism given him by Dr. J.M. deMan.

To the National Research Council of Canada for three times awarding a studentship and the Canadian Dairy Industries and Suppliers' Association for their first yearly award, the author wishes to express sincere thanks.

The writer would also like to pay special tribute to his wife, Lise, whose constant encouragement and unfailing patience have done much to make this work possible.

ABSTRACT

This investigation was undertaken to study the glyceride structure and composition of milk fat triglycerides and especially the relationship between the position and chain length specificity of pancreatic lipase.

The rate of hydrolysis of simple triglycerides, olive oil and milk fat was determined. It was found that pancreatic lipase hydrolyzes short chain fatty acid triglycerides more rapidly than long chain fatty acid ones.

When the pattern of pancreatic hydrolysis of tricaprylin was determined, it was found to be a stepwise hydrolysis yielding dicaprylin and monocaprylin, as was found with long chain fatty acid glycerides. Under similar conditions of digestion, a large amount of glycerol (7.6%) was formed, and the proportions of the 1-isomer were as high as 50.4%.

When an equimolar mixture of triolein and tricaprylin was hydrolyzed by pancreatic lipase, approximately 2 moles of caprylic acid were released after 15% hydrolysis of the total ester groups present and more than 3 moles after one third of total hydrolysis.

Genuine and randomized milk fat was hydrolyzed under optimum conditions of digestion. Evidence was obtained for a preferential attack of pancreatic lipase on milk fat glycerides containing short chain fatty acids. The

generally proposed theory that milk fat contains the short chain fatty acids predominantly at the external positions on the glyceride molecules seems incorrect. Capric, lauric, myristic and palmitic acids were predominantly located at the internal, stearic and oleic acids at the external positions on the glycerides of milk fat.

The pattern of myristic, palmitic, stearic and oleic acids does not follow the same trend in genuine and randomized milk fat. This difference in the fatty acid composition showed that the distribution of fatty acids within the glycerides of milk fat is specific.

Milk fat was found to contain from 4.4 to 6.6% of diglycerides. The small amounts of free fatty acids present preclude the possibility that the diglycerides are the result of lipolysis and it is suggested that they are formed in the synthesizing cells of the mammary gland.

The fatty acid composition of the natural diglycerides was similar to that of diglycerides obtained from pancreatic lipase hydrolysis of the milk fat. The relatively high concentration of short chain fatty acids in the diglycerides does not favour the mechanism of milk fat triglycerides synthesis proposed by Patton and McCarthy.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

THE SPECIFIC DISTRIBUTION OF FATTY ACIDS IN THE GLYCERIDES OF MILK FAT

INTRODUCTION

The distribution of the fatty acids in triglyceride molecules of natural fats has been investigated mainly by classical techniques such as ester fractionation, chromatography, fractional crystallization and countercurrent distribution. Results of these methods indicated little agreement about the distribution of the fatty acids, except as to the prevalence of mixed glycerides.

Several types of arrangement, for example, random, even and restricted random, have been suggested and defended. In 1956, Mattson and Beck (65) described a method of enzymatic hydrolysis, based on the specificity of pancreatic lipase toward the primary ester groups of triglycerides. This provided a new method for the study of glyceride structure, and a specific attachment of fatty acids at each of the hydroxyls of the glycerol molecule was proposed.

These workers suggested that there exists a specific distribution of fatty acids within the glycerides of natural fats.

Since then, the specificity of lipase for fats has, to some extent, been taken for granted. It has been established that the hydrolytic action of pancreatic lipase is specific for the cleavage of ester linkages at the 1 and 3 positions of triglycerides rather than for the chemical nature of the fatty acids at these locations. Whatever their chain length (from C-12 to C-18) and unsaturation (from 0 to 2 double bonds) may be, the external chains are preferentially hydrolyzed over the internal chain.

The question as to whether lipase has any chain length specificity toward the short chain fatty acids (from C-4 to C-10) has never been answered satisfactorily. Until this question is answered, however, it will be impossible to use pancreatic lipase as a tool for determining the glyceridic structure of milk fat, which contains at least 28 major different fatty acids (36) capable of existing in nearly,

$\left[\frac{(28)^3 + 3(28)^2 + 2(28)}{6}\right]$ = 4060 quite distinct combinations, each of which is a specific triglyceride. The high content of short chain fatty acids makes milk fat unique among natural fats.

The purpose of this investigation was to study the glyceride structure and composition of milk fat together with the applicability of pancreatic lipase hydrolysis to better understand the biochemical mechanism for the synthesis of bovine milk fat.

REVIEW OF THE LITERATURE

Several hypotheses on glyceride structure of natural fats are reported in the literature. It is rarely mentioned by research workers what types of distribution of fatty acids they are dealing with. For instance, let us take a fat containing both saturated (S) and unsaturated (U) fatty acids and distribute them among two mixed triglycerides GS_2U and GU_2S. We can obtain the four following forms which are chemically indistinguishable:

SUS, SSU, USU, UUS.

We do not know which of the four structures occur in the triglycerides. In other words, there are two concepts of glyceride structure: (a) the distribution of fatty acids among the triglycerides, and (b) the distribution of fatty acids within the triglycerides.

A. THE DISTRIBUTION OF FATTY ACIDS AMONG THE GLYCERIDES OF NATURAL FATS.

1. Account of the different hypotheses

(a) The random vs. specific distribution

According to the random scheme, any type of

fatty acid may occupy any position in any glyceride (59, 79). This type of arrangement can be easily obtained by esterification of glycerol with fatty acid or interesterification of glycerides. Results obtained by X-ray diffraction studies (86) among other techniques, favour the specific hypothesis in preference to the random hypothesis.

(b) The even distribution

According to this pattern (39: pp 15-18), each fatty acid present to the amount of 0 to 33% must appear only once in an increasing number of triglyceride molecules; every fatty acid present from 33 to 66% must appear twice in an increasing number of triglyceride molecules; finally, simple triglycerides formed, and produced as little as possible, when the proportion of any one fatty acid exceeds 66%.

The hypothesis of Hilditch and his collaborators (34, 39: pp 18 - 21 and 419 - 421) was substantiated in the case of vegetable seed fats but not so well in the case of fruit-coat fats, and not at all in the case of animal fats.

(c) The partial random distribution

Contrary to the even distribution, a fatty acid present less than 33% can occasionally appear twice in the same triglyceride molecule. Doerschuk and Daubert (30) were only able to apply this kind of arrangement in the case of corn oil.

(d) The restricted random distribution

A recent hypothesis of acyl group partition has been suggested by Kartha in the years 1949-50. The results (51, 52, 53) indicated that the fatty acids are distributed according to the laws of chance except that the content of fully saturated glycerides (GS_3) is limited to the amount which can remain fluid in vivo in order to facilitate the fat transportation in animal organisms. Indeed, this kind of distribution would require a rather complicated mechanism for the regulation of triglyceride syntheses (97).

(e) The modified restricted random distribution

The principles of this hypothesis, proposed

by Youngs (103) is a random attachment of the fatty acids at each stage of glyceride synthesis in the order of 1, 2 and 3 with an intramolecular re-arrangement to a preferred form. Unfortunately, Youngs did not supply any proof to show an intramolecular re-arrangement to occur at the 1,2-diglyceride level of fat formation.

This glyceride structure combines aspects of the random distribution of fatty acids among the triglycerides and the specific distribution of fatty acids within the triglycerides.

(f) Vander Wal's hypothesis

In 1960, Vander Wal (97) elaborated a procedure for calculating the fatty acid distribution in natural fats. The method is based on the following two assumptions (98): "that whatever proportions of saturated acyl groups (S) and unsaturated acyl groups (U), are dispersed among the 1-positions, the 2-positions and the 3-positions respectively, of the triglycerides, they are distributed therein at random; that the 1- and 3-positions are occupied

by identical proportions of S and U".

There may be a random distribution involving the 1- and 3-positions but not the 2-position: in lard, for instance, the saturated acids are predominantly in the internal position of mixed glycerides (86).

The hypothesis of Vander Wal is another plausible way to combine the two concepts of glyceride structure. The results obtained by calculating according to this hypothesis in terms of the percentages of the glyceride types GS_3, GS_2U, GSU_2, GU_3 and the isomeric forms, more accurately explained the glyceridic structure of many natural fats, milk fat included (2), than any of the older hypotheses.

2. A critical discussion of the hypotheses, particularly Vander Wal's

The lack of appropriate techniques to isolate the triglyceride types gave rise to a discussion which does not favour the above hypotheses, particularly Vander Wal's hypothesis in the case of natural

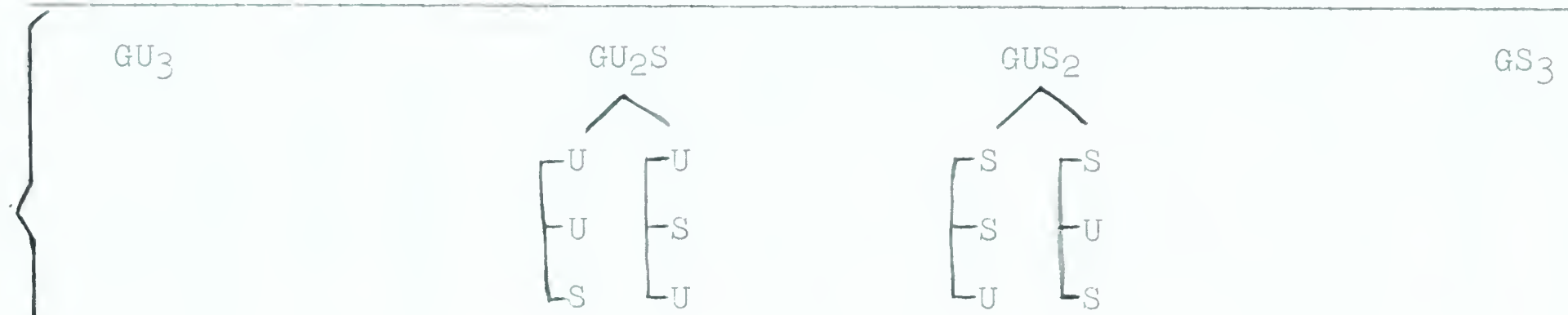
fats containing short chain fatty acids. Bovine milk fat, for instance, has an average molar fatty acid composition of 33% U, 57% S and 10% B (B stands for short chain fatty acids). It is well known that the physical properties of unsaturated and short chain fatty acids are very much alike. For example, the glyceride classes GU_3 and GB_3 would have a low melting point in contrast with GS_3 and would be very difficult to separate from each other. Moreover, the glyceride classes GS_3 and GB_3 can be theoretically distinguished on a chemical basis. Therefore, instead of distributing only saturated and unsaturated fatty acids in the four general classes of glycerides (GU_3, GU_2S, GUS_2, GS_3) we would incorporate in addition, a short chain fatty acid which would give rise to ten distinct classes. In addition, if we take into consideration the isomeric forms of GU_2S and GUS_2, the presence of short chain fatty acids would increase the number of glyceride classes to eighteen possible types (Table 1).

Consequently, the distribution of fatty acids in natural fats such as bovine milk fat, goat milk fat,

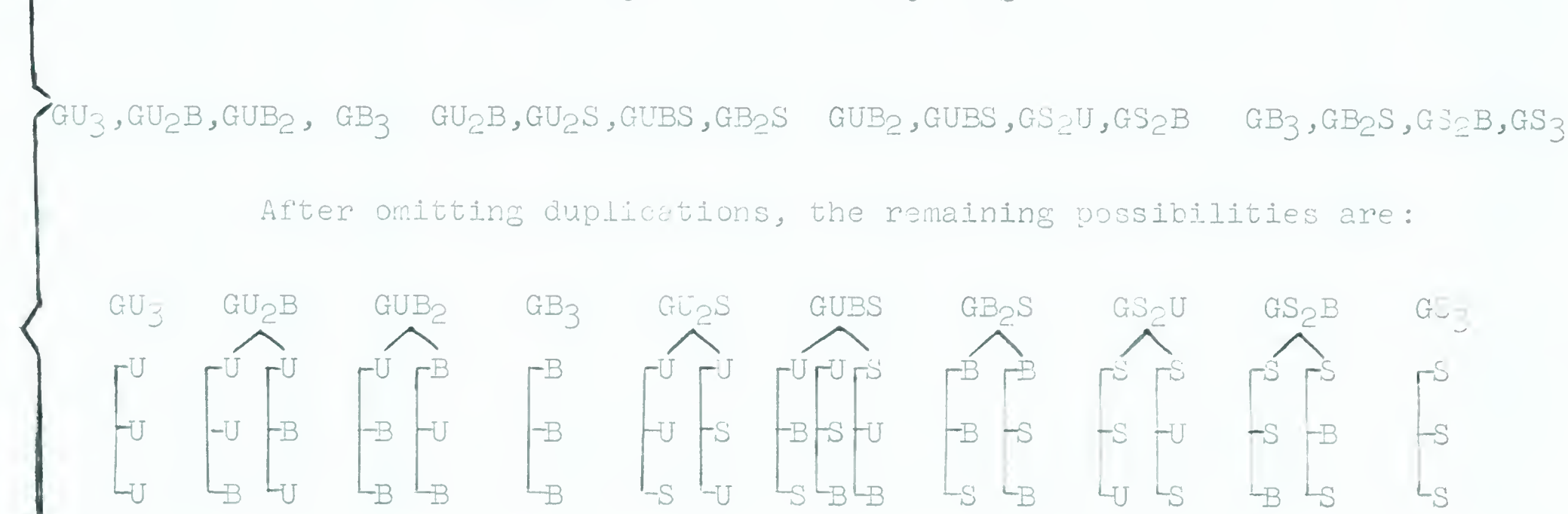
coconut oil and palm-kernel oil which have appreciable percentages of butyric, caproic, caprylic or capric acids, could not be predicted by any of the above hypotheses unless they were substantially modified and extended.

Table 1. TRIGLYCERIDE TYPES AND ISOMERIC FORMS PROPOSED (a) BY THE INVESTIGATORS OF THE PREVIOUS HYPOTHESES (b) BY THE AUTHOR IN THE CASE OF NATURAL FATS CONTAINING SHORT CHAIN FATTY ACIDS

(a)



(b)



S stands for saturated, U for unsaturated, B for short chain fatty acids from C-4 to C-10, E for glycerol backbone and G for glyceride class.

B. THE DISTRIBUTION OF FATTY ACIDS WITHIN THE GLYCERIDES OF NATURAL FATS, PARTICULARLY BOVINE MILK FAT.

1. Account of the hypotheses

A new approach to elucidate the fatty acid distribution in the glycerides of natural fats was disclosed by Mattson and Beck (68) who found that long chain acyl groups in the 1- and 3-positions of triglycerides molecules can be selectively removed by digestion of the fat with pancreatic lipase. Analysis of the 2-monoglycerides and 1,2-diglycerides enabled them to conclude that the acyl groups are specifically distributed within the glycerides of natural fats. The results of this enzymatic technique, applied by several research workers (8, 29, 67, 71, 90, 92) do not favour the random pattern (59, 79).

2. Applicability of pancreatic lipase hydrolysis

(a) Positional specificity of pancreatic lipase

It was demonstrated by several investigators that di- and monoglycerides accumulate during in

vitro hydrolysis by pancreatic lipase (1, 3, 4, 25, 26, 27, 33, 93, 99). Most of this work, however, was carried out with compounds which could not be considered typical of eliminating the positional specificity of pancreatic lipase. A series of experiments were carried out with the four possible glycerides of palmitic (P) and oleic (O) acids (POP, OPP, OPO, POO), which were hydrolyzed by pancreatic lipase until a predetermined proportion of fatty acids was liberated(67, 68, 90, 91). The fatty acid composition of the partial glycerides, resulting from lipolysis, was then identified and calculated. All results established that the proportion of the fatty acids found in the diglyceride fractions were in a similar ratio and that most of the fatty acids found in the monoglycerides belonged to the internal position. Additional experiments carried out by Borgström (8, 11), Mattson et al. (68, 70) and Savary and Desnuelle (90) among others (94, 96), indicated that the long chain fatty acids, namely the saturated ones from C-12 to C-18 (lauric, myristic, palmitic and stearic) and the most common C-18 unsaturated

fatty acids (oleic and linoleic) are hydrolyzed at about the same rate by pancreatic lipase.

However, since the observation of the presence of as much as 20 - 30% of 1-monoglycerides at the end of the hydrolysis, the question remains whether the fatty acid at the internal position of the triglyceride molecules are slowly split or not at all by the enzyme (11, 70, 72, 90). An aqueous suspension of synthetic 2-monoolein at 37°C and pH 8.0 isomerized so rapidly that equilibrium at 88 - 92% 1-monoglyceride and 8- 12% 2-monoglycerides is reached within a few minutes (66). The surprising fact is to find only 20 - 30% of 1-monoglycerides in the total monoglyceride fraction resulting from the in vitro hydrolysis (8, 67, 70, 90). This difference in the monoglyceride equilibrium may be explained by the stabilizing effect of free fatty acids.

It appears, then, that some internal chains may be split after isomerization to the external positions, but it is suggested also (16, 17, 18,

29, 71, 73, 88, 92, 103) that the positional specificity of pancreatic lipase is almost absolute. Therefore, the main pathway of the in vitro lipolysis can be summarized as follows:

Triglyceride ⟶ 1,2-diglyceride ⟶ 2-monoglyceride

The formation of free glycerol is difficult unless very large amounts of enzyme and bile salts are used (8, 19).

(b) Chain length specificity of pancreatic lipase

The next step in the study of substrate specificity of pancreatic lipase would be to answer whether lipase has any chain length specificity toward the so-called short chain fatty acids, from C-4 to C-10 (butyric, caproic, caprylic and capric). The literature on this specific problem is rather restricted.

In 1951, Desnuelle (24) mentioned that the pancreatic lipase digestion of tributyrin is faster than triolein. Ten years later, Entressangles et al. (31) found that both purified and crude pancreatic lipase preferentially released butyric acid from the diglyceride, glyceryl-1-palmitate-

3-butyrate. The molar composition of the free fatty acids was 72% butyric acid and only 28% oleic acid after 10% of hydrolysis of the total ester groups. In 1962, Clément et al. (14) observed some anomalies in the composition of the di- and monoglycerides derived from 2-butyryl-dipalmitin and demonstrated a slight preferential hydrolysis of the butyric acid.

Thus, it is likely that pancreatic lipase splits esters of short chain fatty acids at the internal position of a triglyceride molecule. Therefore, the chain length specificity of pancreatic lipase should be studied in detail .

(c) The glyceride content and the fatty acid composition of fresh bovine milk fat diglycerides compared with partial glycerides from pancreatic lipase hydrolysis.

Milk lipids consist essentially of glycerides together with small amounts of phospholipids, cholesterol, cholesterol esters, vitamin A esters,

free fatty acids and other minor components (46: pp 38, 40, 41). It is usually assumed in the literature (83, 46: p 35) that the glycerides consist entirely of triglycerides.

There is some evidence, however, that small quantities of monoglycerides (48, 49, 50, 76: p 492) and possible diglycerides (43, 44, 45, 50) are present in the mixed triglycerides of normal milk fat. But, as yet, no investigations on the content and analysis of diglycerides in fresh bovine milk fat have been published. This information combined with a study of pancreatic lipase hydrolysis of fresh milk fat are reported herein.

EXPERIMENTAL PROCEDURE

The influence of the chemical nature of the acyl groups on the positional specificity of pancreatic lipase has been investigated by several techniques. The first one has involved the use of a series of simple triglycerides and a comparison of the relative rates at which the enzyme splits off different fatty acids by a fixed amount of pancreatic lipase under a given set of conditions. The second technique involved the stepwise hydrolysis of a simple triglyceride of a short chain fatty acid. The third experimental approach was the analysis of the molar composition of the free fatty acid and glyceride fractions from the hydrolysis of an equimolar mixture of simple triglycerides of long and short chain fatty acids. The fourth procedure involved a comparative study of the hydrolysis of milk fat before and after interesterification. This treatment brings about a complete randomization of the fatty acids on all possible positions. The fatty acid composition of the different glyceride fractions will consequently indicate the rate at which the

various fatty acids have been split off.

The enzymatic technique has been applied on fresh milk fat to compare the fatty acid composition of the diglycerides of fresh milk fat with those obtained after lipolysis by pancreatic lipase.

A. PREPARATION OF MILK FAT

1. Fresh milk fat

Milk fat was obtained from fresh raw milk by repeated creaming and washing of the cream with distilled water in a refrigerator. The washed cream was churned in a Waring Blendor and the butter granules melted. Then, the fat was filtered at 45°C through Whatman No. 1 filter paper and dried under vacuum. An alternative procedure for the preparation of milk fat was also used. It involved washing the butter granules with warm water according to the procedure described by deMan (22). All the samples were stored at about -25°C in the freezer.

2. Interesterified milk fat

The interesterification (randomization) of milk

fat glycerides was accomplished by treatment with 0.2% sodium methylate for 1 hr at 50°C and subsequent neutralization with an excess of citric acid to inactivate the catalyst according to the method outlined by deMan (22).

B. ENZYME SOURCE

Pancreatic lipases of various mammals such as cattle (55), dog (63), rat (6, 7, 9, 10, 32, 70, 87) and human (35) show the same positional specificity as pig pancreatic lipase. The pig lipase has been chosen in this investigation over the other animal lipases because of its availability and wide-spread use.

Pancreatic lipase (Steapsin), obtained from Nutritional Biochemicals Corporation is a crude enzyme preparation obtained from hog pancreas (80). As similar results were obtained with this as with purified preparations (63, 64, 65, 84, 101, 102) the product as supplied was used as such for all experiments.

C. CONDITIONS OF HYDROLYSIS

The lipolysis conditions, used in the study of triglycerides other than milk fat glycerides, were essentially those described by Desnuelle *et al.* (27), except that no buffer solution was added. Unless otherwise indicated, the digestion mixture consisted of 0.3 ml of a 0.15% aqueous solution of bile salts (Difco) as emulsifier, 1.0 ml of a 0.45% aqueous calcium chloride solution as activator, 156 mg of pancreatic lipase and 2 g of triglycerides.

Optimum concentration of calcium ions, hydrogen ions, enzyme and substrate together with the optimum temperature, were determined before setting the conditions of milk fat hydrolysis by pancreatic lipase. On the basis of exploration work pancreatic lipase lipolysis was carried out in a digestion mixture containing bile salts, as emulsifier; 2.0 ml of a 4% aqueous solution of calcium chloride; 700 mg of steapsin and 5 g of milk fat. Sufficient water was added to bring the volume to about 75 ml. The mixture was violently agitated and the pH kept at 8.0 - 8.5 by addition of 1 N NaOH during the hydrolysis, at 37° C.

After hydrolysis had progressed to a predetermined extent, the enzyme action was stopped by addition of 10 ml ethanol and by heating the mixture to 70°C for a few minutes.

D. EXTRACTION OF GLYCERIDES FROM THE REACTION MIXTURE

The glyceride and free fatty acid fractions were extracted with four 250 ml volumes of petroleum-ether-diethyl-ether(50/50). The ether solution was washed five times with 50 ml of 1% sodium bicarbonate to remove fatty acids. The remaining neutral glyceride solution was dried with anhydrous sodium sulfate, filtered and the solvent evaporated under vacuum.

When low molecular weight glycerides, for instance monobutyrin, which is relatively soluble in water and difficult to extract quantitatively from an aqueous solution, was expected to be present in the digestion mixture, the lipid extraction method was modified by evaporating the hydrolysate until dryness and extraction with the petroleum ether-diethyl ether mixture, followed only by drying with anhydrous sodium sulfate. However, similar recovery was obtained with these two extraction procedures.

E. SEPARATION OF GLYCERIDES BY COLUMN CHROMATROGRAPHY

Chromatography on silicic acid is widely used for the separation of tri-, di- and monoglycerides (5, 12, 40), but the separations are not always reliable even if particular care is exercised. Separations can be achieved in much shorter times with smaller volumes of eluting solvents by using Florisil (magnesium silicate) which has the further advantage that free fatty acids are eluted after the various glyceride fractions and this eliminates the possibility of their overlapping with tri- or diglycerides (40).

The chromatographic method which was used is similar to the one described by Carrol (13). The Florisil was deactivated with 7% (v/w) of water and the charge of each column was 18 g. The amount of glycerides applied was 50 - 150 mg and elution was done with 100 ml of 15% diethyl ether in hexane for triglycerides, 90 ml of 50% diethyl ether in hexane for diglycerides and 100 ml of 3% methanol in diethyl ether for monoglycerides. The free fatty acids, wherever they were eluted, were obtained with 100 ml of 4% acetic acid in diethyl ether.

F. CHARACTERIZATION OF LIPID MATERIALS

Thin-layer chromatography (61, 62, 85) was the method used to check the purity of the glyceride fractions eluted from the Florisil columns. Unless otherwise indicated, the lipid fractions were dissolved in petroleum ether, spotted on plates which were coated with Silica Gel G as adsorbent, and developed with petroleum ether, diethyl ether, acetic acid, 90-10-1. A developing time of 25 - 35 minutes was sufficient to provide a separation of the components. The spots were made visible with iodine vapour (95).

Sometimes, complementary determinations such as the hydroxyl and acid values (77) were carried out as additional tests to check some of the various lipid materials used in this study.

G. DETERMINATION OF MONOGLYCERIDES AND GLYCEROL

The presence of 1-isomer in the 2-monoglyceride fractions resulting from hydrolysis by pancreatic lipase, was determined by the periodic acid method

described by Martin (56). The glycerol content was analyzed by the procedure of Lambert and Neish (58).

H. PREPARATION AND ANALYSIS OF THE METHYL ESTER BY GAS LIQUID CHROMATOGRAPHY

Methyl esters were prepared by refluxing a lipid sample for 10 minutes with two volumes of 0.025 N potassium methylate in methanol, as outlined by deMan (21). Then the pentane solution of the methyl esters was injected directly on to the column of a Wilkens Aerograph, model 110 with thermal conductivity detector.

Because of the possible loss of the volatile and water soluble methyl esters of the short chain fatty acids, methylation was also accomplished in a freeze drying bulb. Once sealed, the bulb containing the reactants was heated 1 hour in an oven at 60°C. A clearing of the system, which is initially in two phases, was the sign of complete methylation. The solution of methyl esters in methanol was injected without further treatment at 70°C into the column of an F & M, model 720, dual column programmed temperature gas chromatograph.

The areas under the peaks were measured by a planimeter and converted to weight percentage methyl esters by using correction factors, established with pure compounds under identical conditions (23).

RESULTS

A. RATE OF HYDROLYSIS OF VARIOUS GLYCERIDES

The comparative rates of hydrolysis of fresh milk fat, neutral olive oil and simple triglycerides were determined. During the digestion period (10 min) at 37°C and 45°C, the mixture was continuously agitated and a pH of 8.5 was maintained by the addition of 1 N NaOH. The activity of pancreatic lipase with various triglycerides, olive oil and milk fat is presented in Table 2.

Table 2. RELATIVE RATES OF HYDROLYSIS OF MILK FAT, OLIVE OIL AND SIMPLE TRIGLYCERIDES

Substrate	% hydrolysis [a]	
	37°C	45°C
1. Tributyrin (Eastman)	39.8	44.0
2. Tricaproin	29.0	34.5
3. Tricaprylin	41.9	48.1
4. Trilaurin	15.2	14.7
5. Trimyristin (Eastman)	14.8	14.9
6. Triolein (K & K)	14.8	14.8
7. Olive Oil (Berio's)	13.9	13.4
8. Milk fat (winter)	17.2	20.2

a. Extent of hydrolysis in % of the total ester groups present.

The general pattern of the relative rates of hydrolysis are in agreement with the results of Wills (102), except that the highest rate is found for tricaprylin rather than tributyrin. This discrepancy may be explained by the presence of partial glycerides in the tributyrin sample as observed in most of commercial products (Table 3 and Figures 1 & 2).

Table 3. WEIGHT PER CENT OF MONO-, DI-, AND TRIGLYCERIDES (MG, DG AND TG) IN COMMERCIAL GLYCERIDES AS OBTAINED BY CHROMATOGRAPHY ON FLORISIL

Commercial products	Column load	Glyceride content			Recovery[c]
	mg	TG	DG	MG	%
1. Tributyrin[a] (Math.Col.& Bell)	156.6	68.6	29.1	2.3	98.9
" (Eastman)	99.6	66.6	31.0	2.4	97.2
" (Fischer)	106.5	63.6	33.4	3.0	97.0
2. Tricaproin[a] (Eastman)	96.0	65.2	34.8	0.0	98.1
3. Tricaprylin (Eastman)	59.7	95.0	3.7	1.3	101.3
4. Trilaurin (Eastman)	85.3	94.8	4.9	0.3	99.9
5. Trimyristin[a] (K & K)	106.4	90.2	8.8	1.0	100.3
" (Eastman)	95.1	95.5	4.4	0.1	102.0
6. Tripalmitin (K & K)	94.6	85.5	13.3	1.2	100.8
7. Tristearin (Math.Col.& Bell)	118.4	64.9	34.3	0.8	100.4
8. Triolein[a] (K & K)	105.4	91.4	7.7	0.9	101.2
" (Math.Col.& Bell)	152.9	80.3	17.5	2.2	101.3
9. Olive Oil (Berio's)	98.1	92.5	6.0	1.5	100.7
" (Mazola)	78.9	93.4	6.5	0.1	100.6
10. Peanut oil (Planters)	85.2	90.8	9.2	0.0	100.6
11. Milk fat (Winter)	88.7	93.9	6.1	0.0	101.0
12. 1-Mono-n-butyrin[a] (Fischer)	117.5	2.7	32.3	65.0	69.0[b]

a. Further identified by thin layer chromatography (Figures 1 and 2).
b. Low recovery is attributed to the presence of glycerol in the sample.
c. Recovery is the weight per cent of glycerides recovered by column chromatography; the ideal case being 100%.

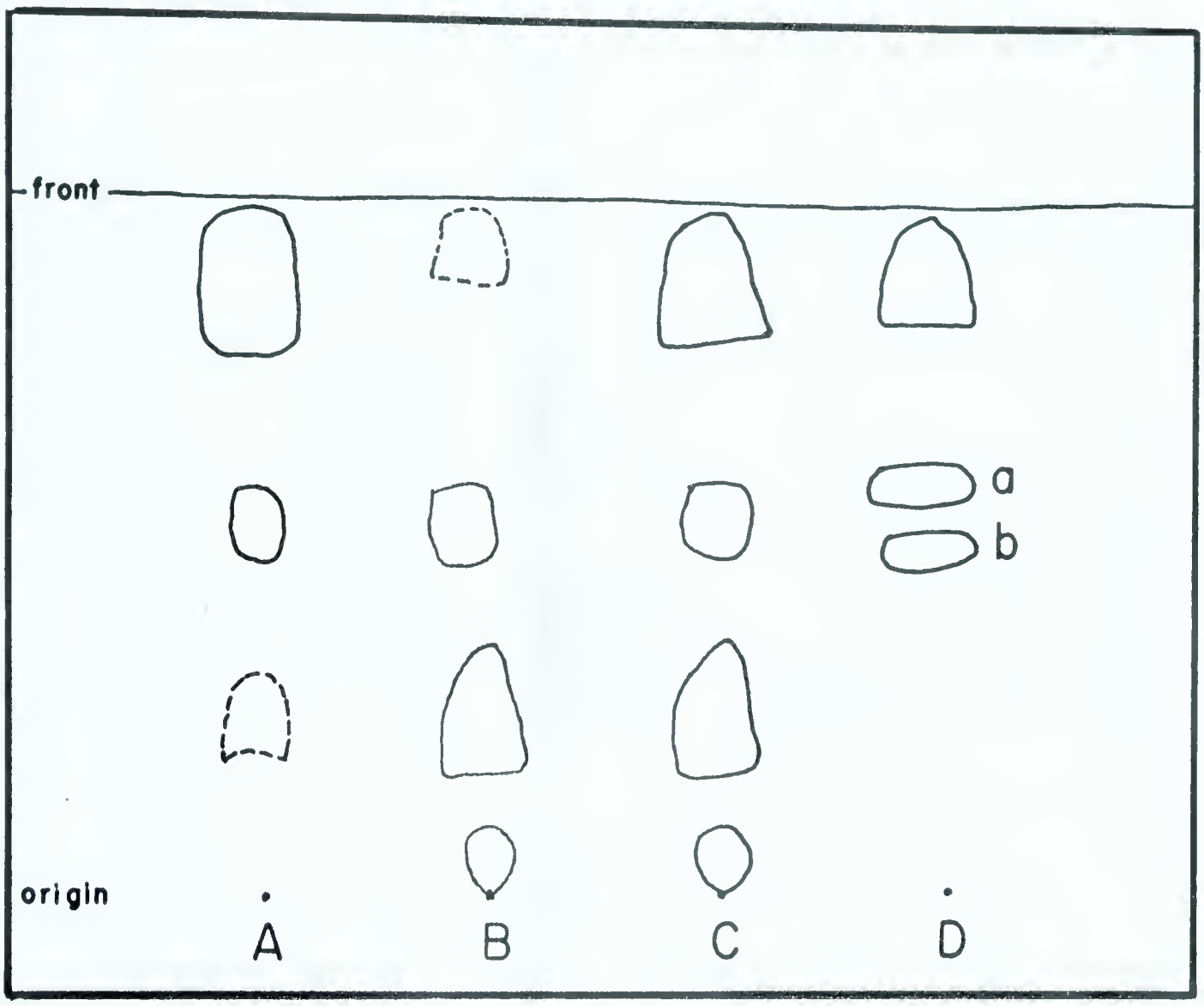


Fig.1. THIN LAYER CHROMATOGRAM OF TRIBUTYRIN, TRICAPROIN AND 1-MONO-n-BUTYRIN.
Samples: A. Tributyrin, B. 1-Mono-n-butyrin, C.Mixture of tributyrin and 1-mono-n-butyrin, D. Tricaproin. Sample size: 5 µl of 1% solution in ethanol.Mobile phase: 45:55 (ethyl ether: petroleum ether; v/v). Stationary phase: Silica Gel no.1 (Fischer). Spot detection:Rhodamine 6 G. Further details: A dashed area indicates a weak spot. From origin to front: glycerol; mono-glycerides; diglycerides: (a) 1,3-diglycerides, (b) 1,2-diglycerides; triglycerides.

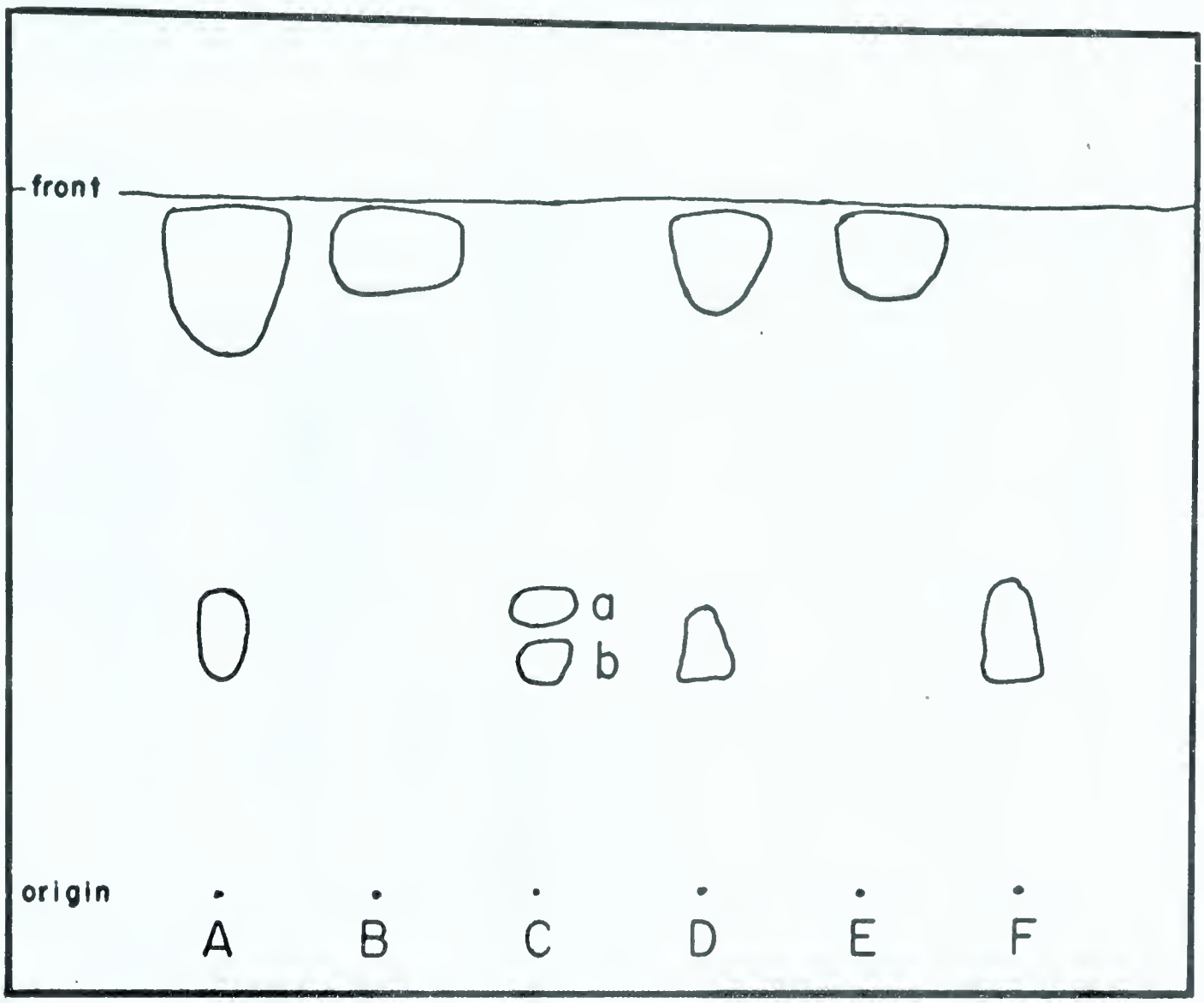


Fig. 2. THIN LAYER CHROMATOGRAM OF TRIMYRISTIN AND OLIVE OIL.
Samples: A. Trimyristin, B.Trimyristin fraction obtained by column chromatography on Florisil, C.Dimyristin fraction, D.Olive Oil, E.Triglyceride fraction, F.Diglyceride fraction. Sample size: 10 µl of 1% solution in petroleum ether. Mobile phase: 30:70 (ethyl ether: petroleum ether; v/v). Stationary phase: Silica Gel no. 1 (Fischer). Spot detection: Iodine vapour. Further detail: The slowest moving diglyceride spot is the 1,2-isomer (83).

B. HYDROLYSIS PATTERN OF TRICAPRYLIN

Several investigators (8, 11, 67, 70, 90, 96) have provided evidence indicating that a long chain fatty acid triglyceride is degraded in vitro to 1, 2-diglyceride and mainly to 2-monoglyceride by the action of pancreatic lipase. This earlier work was limited to glycerides consisting of long chain fatty acids. There was the possibility that the same types of intermediates would not accumulate during the hydrolysis of glycerides of short chain fatty acids. As no data have as yet been reported on the stepwise degradation of a short chain fatty acid triglyceride, the pattern of tricaprylin was determined as follows: (a) hydrolysis with pancreatic lipase, (b) extraction of neutral lipids from the reaction mixture, (c) separation of mono-, di- and triglycerides by column chromatography on Florisil, (d) determination of 1- and 2-monoglycerides by the periodic acid method described by Martin (66). Since monoglycerides do isomerize on Florisil (72), it was necessary to determine the amount of 1-isomers before passing the hydrolysis products through the chromatographic column.

Free glycerol was analyzed by the method of Lambert and Neish (58).

The course of lipolysis of tricaprylin is represented by the data in Table 4.

Table 4. COLUMN CHROMATOGRAPHIC SEPARATION OF NEUTRAL GLYCERIDE FRACTIONS PRODUCED BY PANCREATIC LIPASE HYDROLYSIS OF TRICAPRYLIN AT pH 8.5, AT 37°C.

% Hydrolysis	Weight % glycerides			% Recovery
	TG	DG	MG	
0.0[a]	95.0	3.7	1.3	100.6
10.1	73.0	24.6	2.4	95.2
20.0	49.5	41.8	8.7	96.4
30.1	30.7	54.9	14.4	96.0
40.4	18.1	52.0	30.0	93.4

a. Original sample before hydrolysis

The course of the enzymatic hydrolysis of tricaprylin which is expressed by the concentrations in mole % of the various glyceridetypes at the end of each period of hydrolysis is graphically shown in Figure 3. The discussion of Figure 4 will be seen later on in the text. The percentage hydrolysis was calculated from the volume of 1 N NaOH added during the digestion period and represented the extent of hydrolysis of

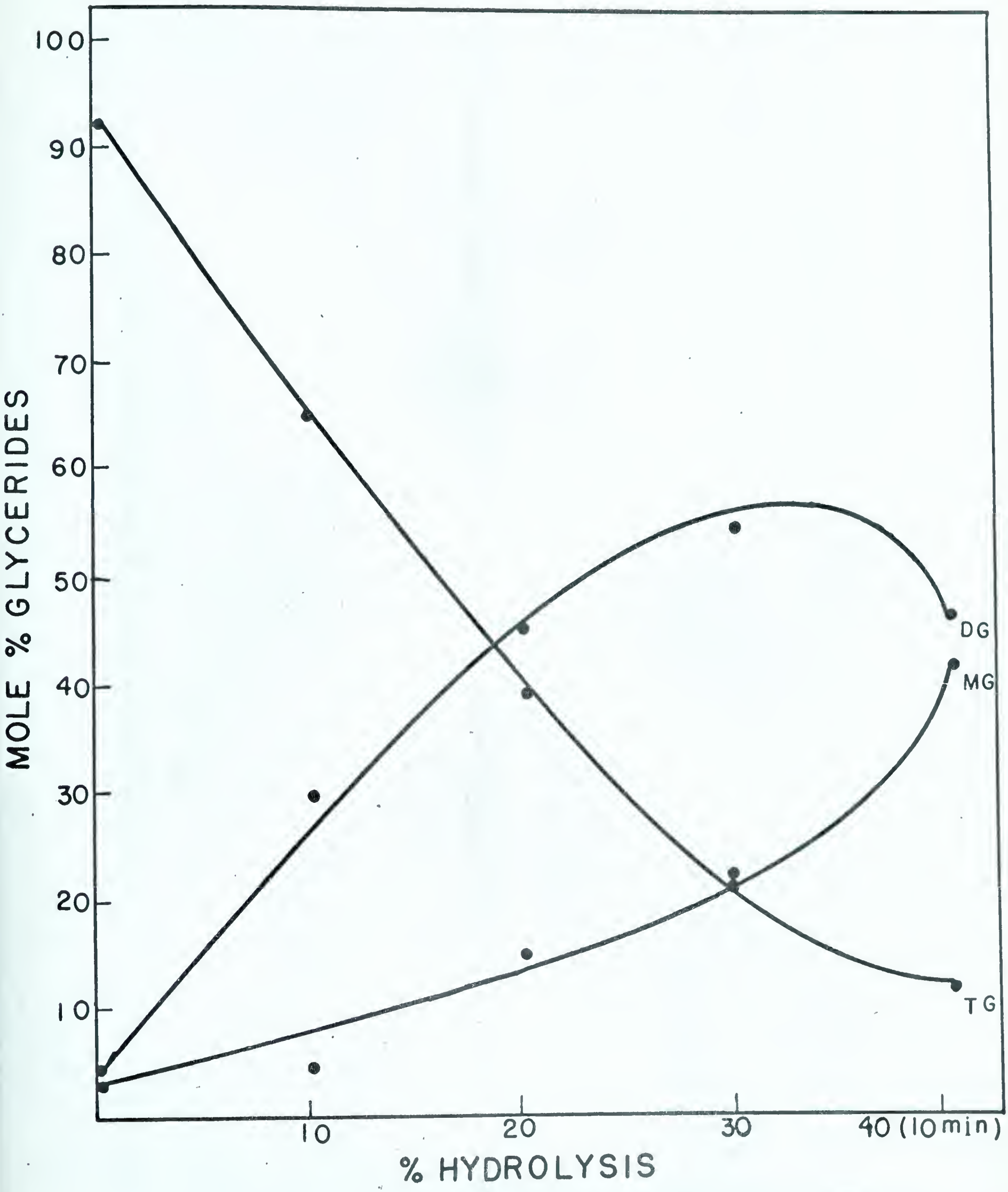


Fig. 3. THE COURSE OF THE ENZYMATIC HYDROLYSIS OF TRICAPRYLIN.

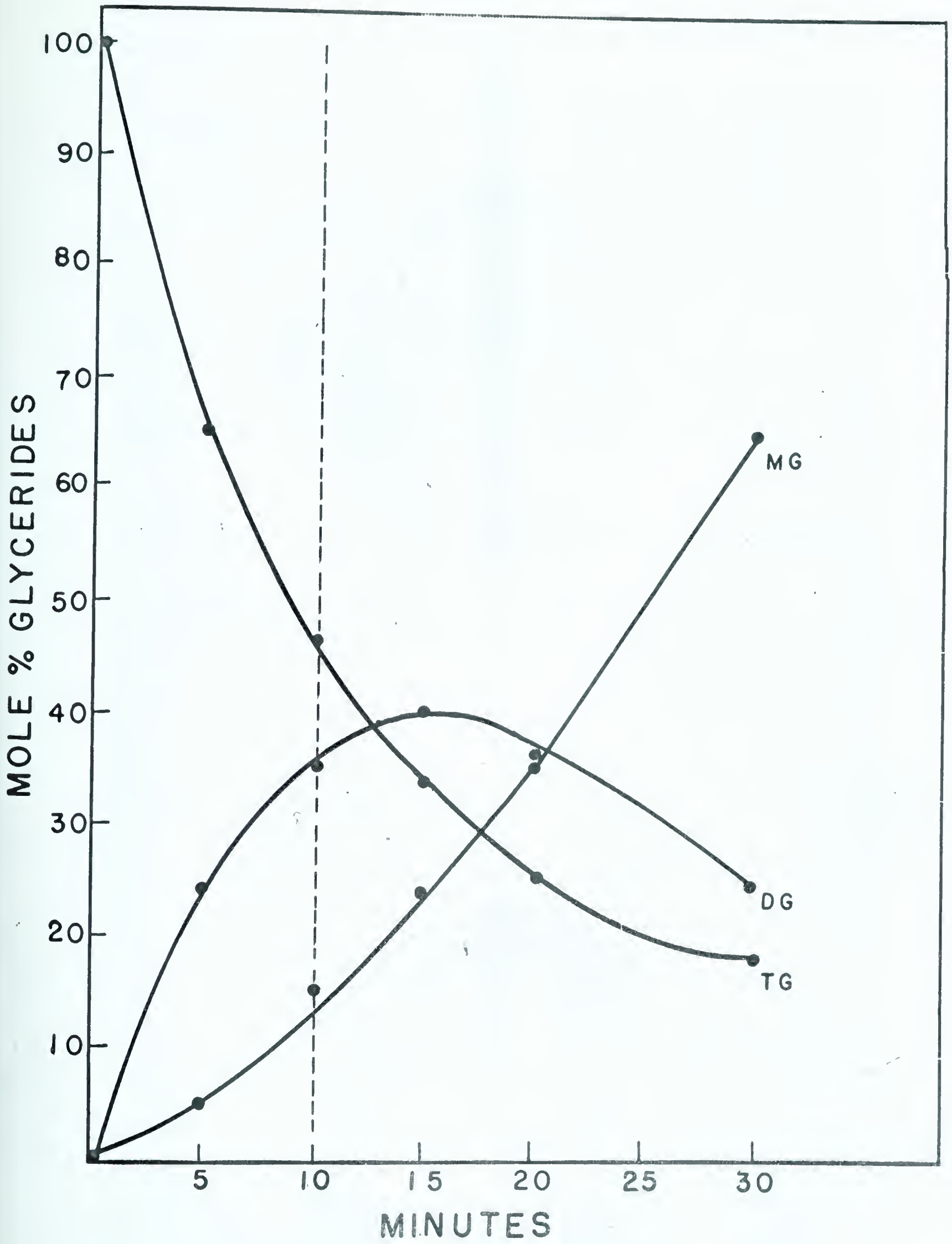


Fig. 4. THE COURSE OF THE ENZYMATIC HYDROLYSIS OF TRIGLYCERIDES (2-OLEOYL-DIPALMITIN) ACCORDING TO MATTSON AND BECK (57).

the total ester groups present. The results of analyses of 1- and 2-monoglycerides are given in Table 5. When the method of Lambert and Neish is applied to pure glycerol (C.P. : assay 99.5% glycerol), Beer's law is followed. These data give a straight line when absorbance is plotted against the concentration of glycerol (Figure 5).

Table 5. DETERMINATION OF MONOGLYCERIDE AND GLYCEROL CONTENT RECOVERED AT THE END OF A 10 MINUTE DIGESTION PERIOD OR ABOUT 40% HYDROLYSIS OF TRICAPRYLIN AT pH 8.5, at 37° C.

	%
1. Free monocaprylin	
Before isomerization	
2-isomer	49.6
1-isomer	50.4
After isomerization	
2-isomer	6.7
1-isomer	93.3
2. Free glycerol	7.6

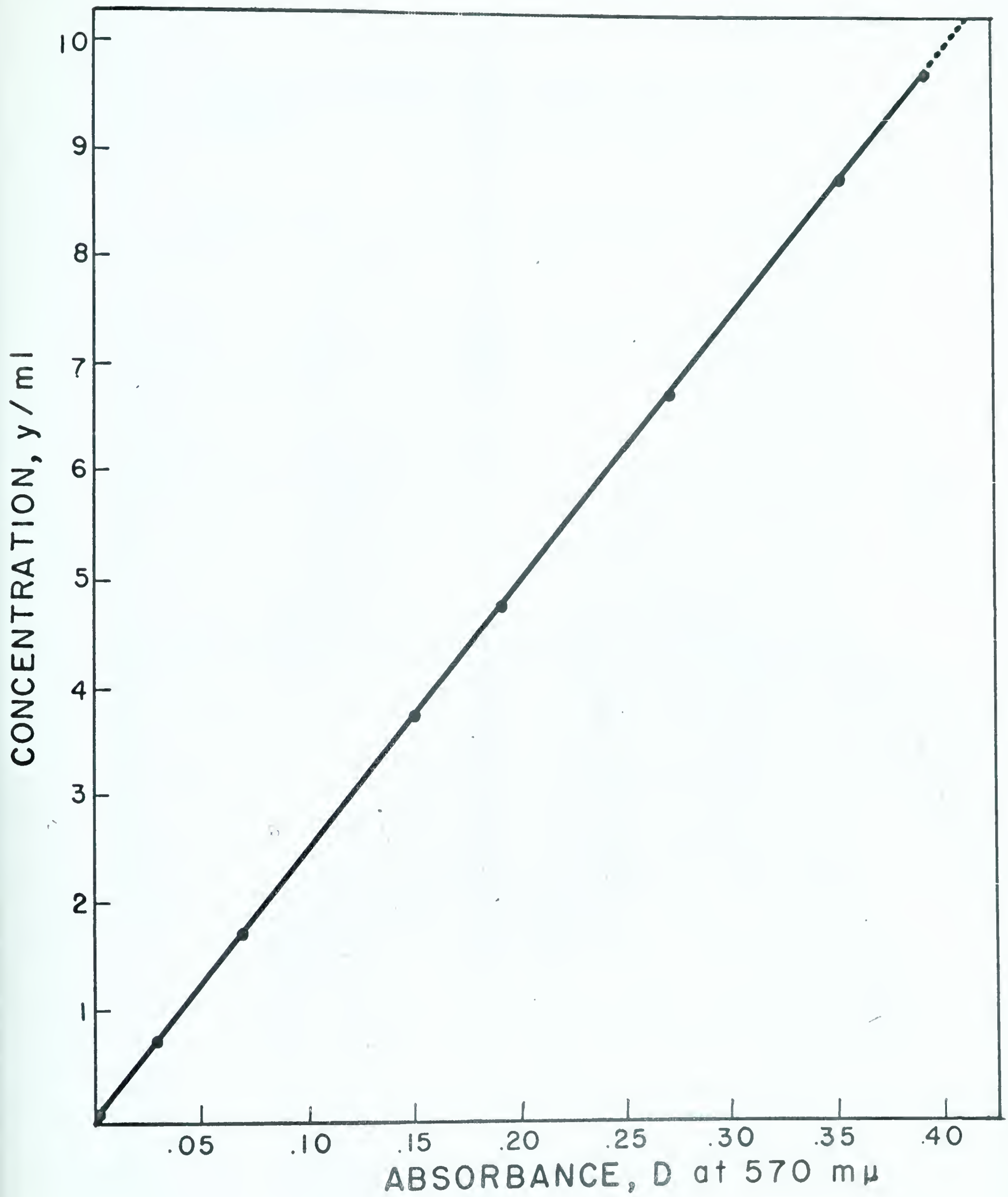


Fig. 5. CALIBRATION CURVE FOR GLYCEROL DETERMINATION

C. RATE OF HYDROLYSIS OF AN EQUIMOLAR MIXTURE OF SHORT AND LONG CHAIN FATTY ACID TRIGLYCERIDES

The methods of digestion and extraction were essentially the same as described before. The fatty acid and glyceride fractions obtained after various extents of hydrolysis of the tricaprylin-triolein mixture were methylated by means of the freeze drying bulb method. The methyl esters were analyzed with a programmed temperature gas chromatograph as described in the experimental procedure.

The figures which are shown in Table 6 are calculated from the data obtained by gas chromatographic analysis of methyl esters of the equimolar mixture.

Table 6. MOLE PER CENT OF FREE CAPRYLIC AND OLEIC ACIDS RELEASED BY PANCREATIC LIPASE AT pH 8.5, at 37°C.

% Hydrolysis	Fatty acids in glyceride fraction [a]		
	Caprylic	:	Oleic
0.0	48.1	:	51.9
15.0	66.0	:	34.0
32.8	76.5	:	23.5
48.8 [b]	78.0	:	22.0

a. Determined as methyl esters by gas liquid chromatography.

b. The acid fraction contained 81.0% caprylic acid and 19.0% of oleic acid esters.

D. RATE OF HYDROLYSIS OF VARIOUS FATTY ACIDS OF MILK FAT BEFORE AND AFTER INTERESTERIFICATION

1. Factors influencing the rate of hydrolysis of milk fat

The conditions of hydrolysis chosen were such as to give maximum rates. The effect of varying the constituents of the digestion mixture was investigated first.

The digestion conditions were those outlined in the experimental procedure. The substrate was a winter milk fat. During the digestion period (15 min) the mixture was continuously agitated and a pH of 8.0 was maintained by the addition of 1 N NaOH. For each analysis, the quantity of alkali added was used as a measure of the rate of hydrolysis.

(a) Effect of electrolyte concentration

The extent of hydrolysis of the total ester groups found after 15 minutes of hydrolysis, when the reaction mixture contained various concentrations of calcium chloride, is shown in Figure 6. In the absence of this electrolyte

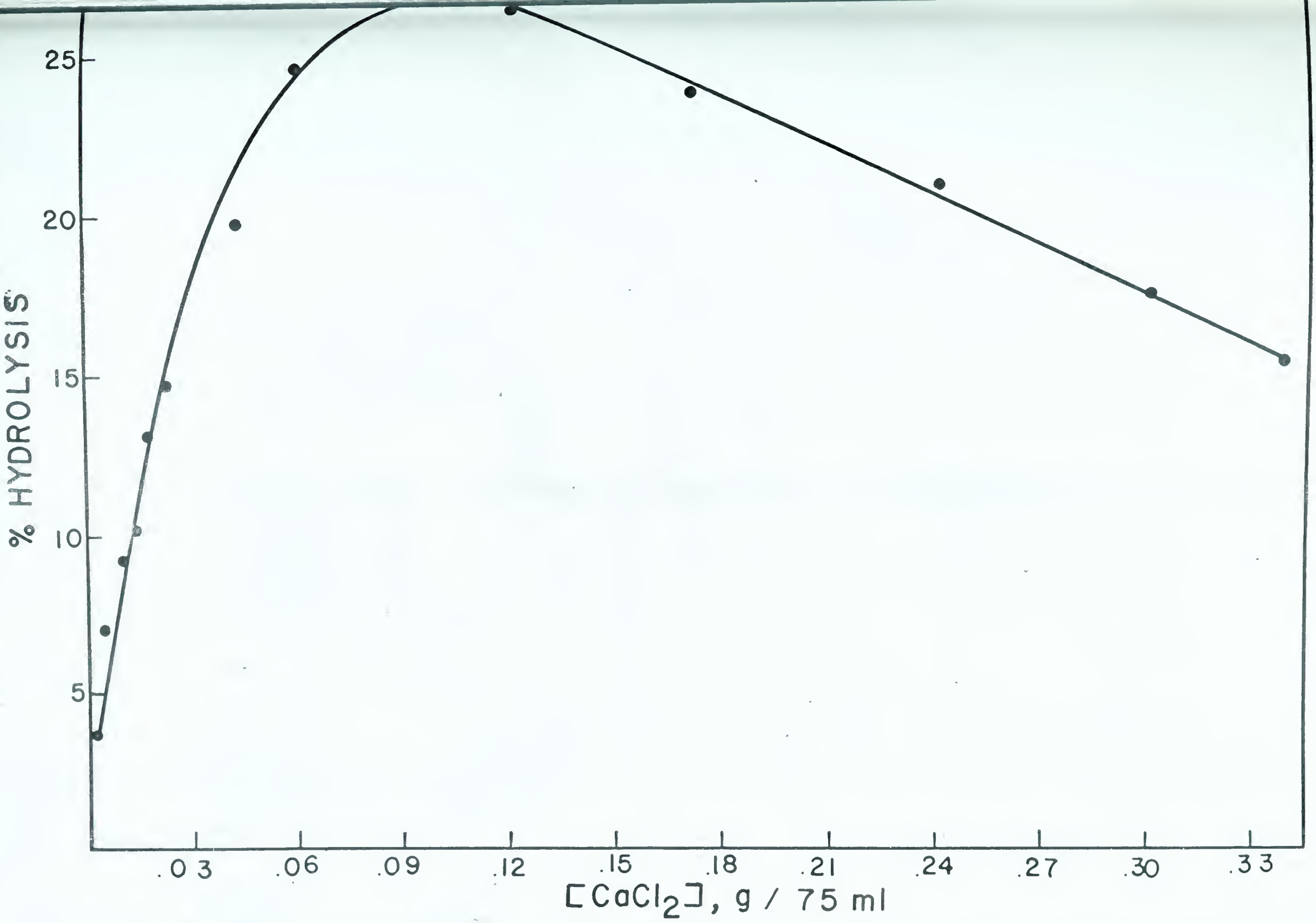


Fig. 6. EFFECT OF ELECTROLYTE CONCENTRATION ON THE RATE OF DIGESTION OF MILK FAT BY PANCREATIC LIPASE

the digestion proceeded quite slowly. As the concentration of electrolyte was increased, the rate of digestion reached a maximum. With further increasing concentration, the emulsion became unstable probably because of a "salting out" effect. The optimum concentration of the activator was in the neighbourhood of 80 mg per 156 mg of enzyme (in 75 ml, total volume).

(b) Effect of temperature

Figure 7 shows the effect of temperature on the digestion of milk fat. The optimum activity of pancreatic lipase was found to be in the range of 45 - 55°C for a digestion period of 15 minutes. No experiments were carried out below 30°C because of the solid state of the substrate at this temperature.

(c) Effect of hydrogen ions

A plot expressing the relationship between the percentage hydrolysis of milk fat against pH is given in Figure 8. Pancreatic lipase was

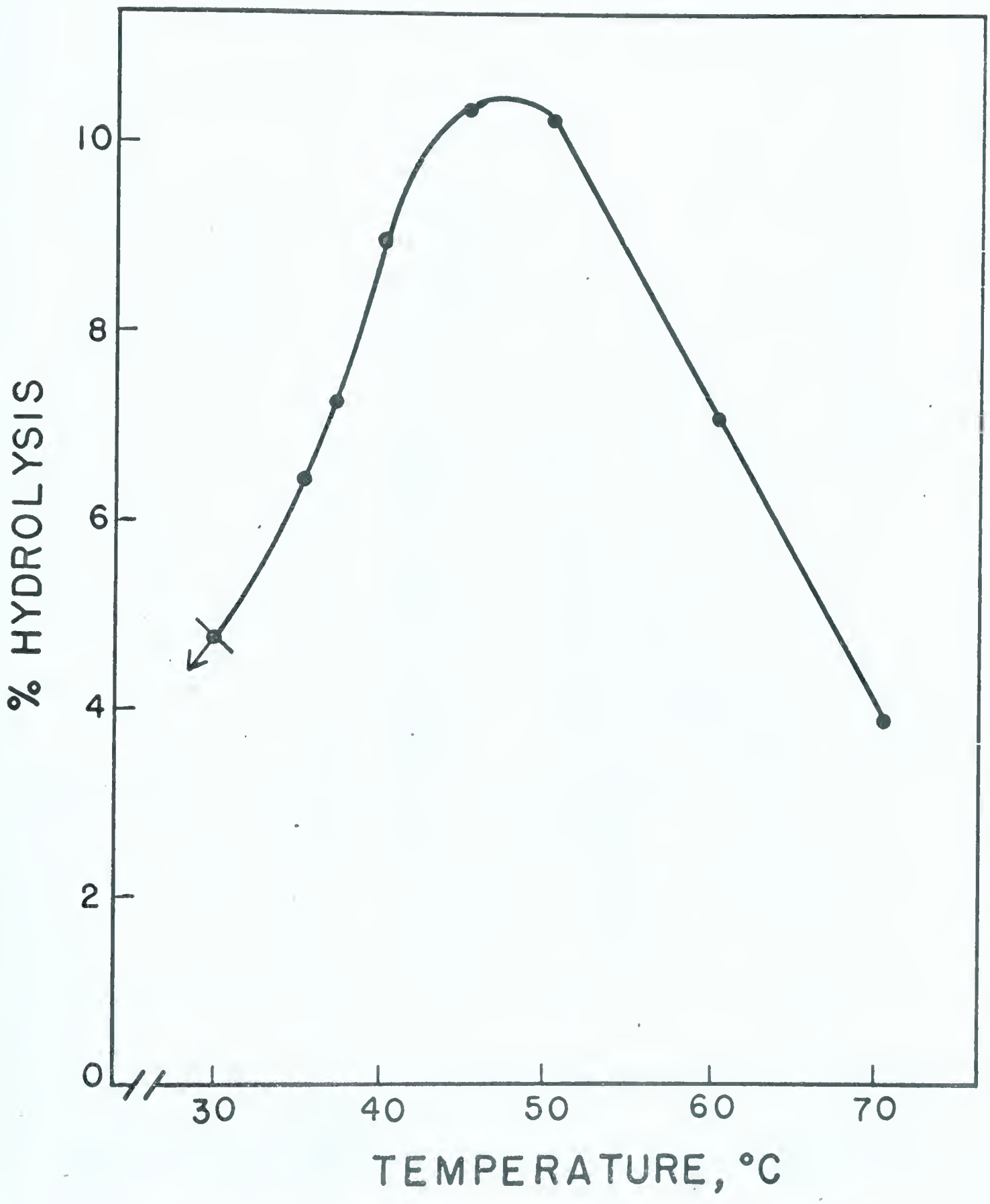


Fig. 7. EFFECT OF TEMPERATURE ON THE RATE OF HYDROLYSIS OF MILK FAT BY PANCREATIC LIPASE

active only over a restricted range of pH values. The optimum pH was found to be in the region of 8.5 to 9. Extremes of acidity and alkalinity led to enzyme inactivation.

(d) Effect of enzyme concentration

The effect of varying the amount of pancreatic lipase on the extent of hydrolysis is shown in Figure 9. The rate of digestion increased as the amount of added enzyme increased. It was apparent that hydrolysis of milk fat was proportional to the concentration of the enzyme between 18 and 780 mg. However, the initial response was not linear as it was observed by Mattson and Beck (67).

(e) Effect of substrate concentration

The influence of substrate concentration on initial reaction velocity ($v = -d[S]/dt$), expressed as the amount of substrate converted per unit of time, is described by means of the curve in Figure 10. After an initial rise, the curve sloped off and finally reached a constant maxi-

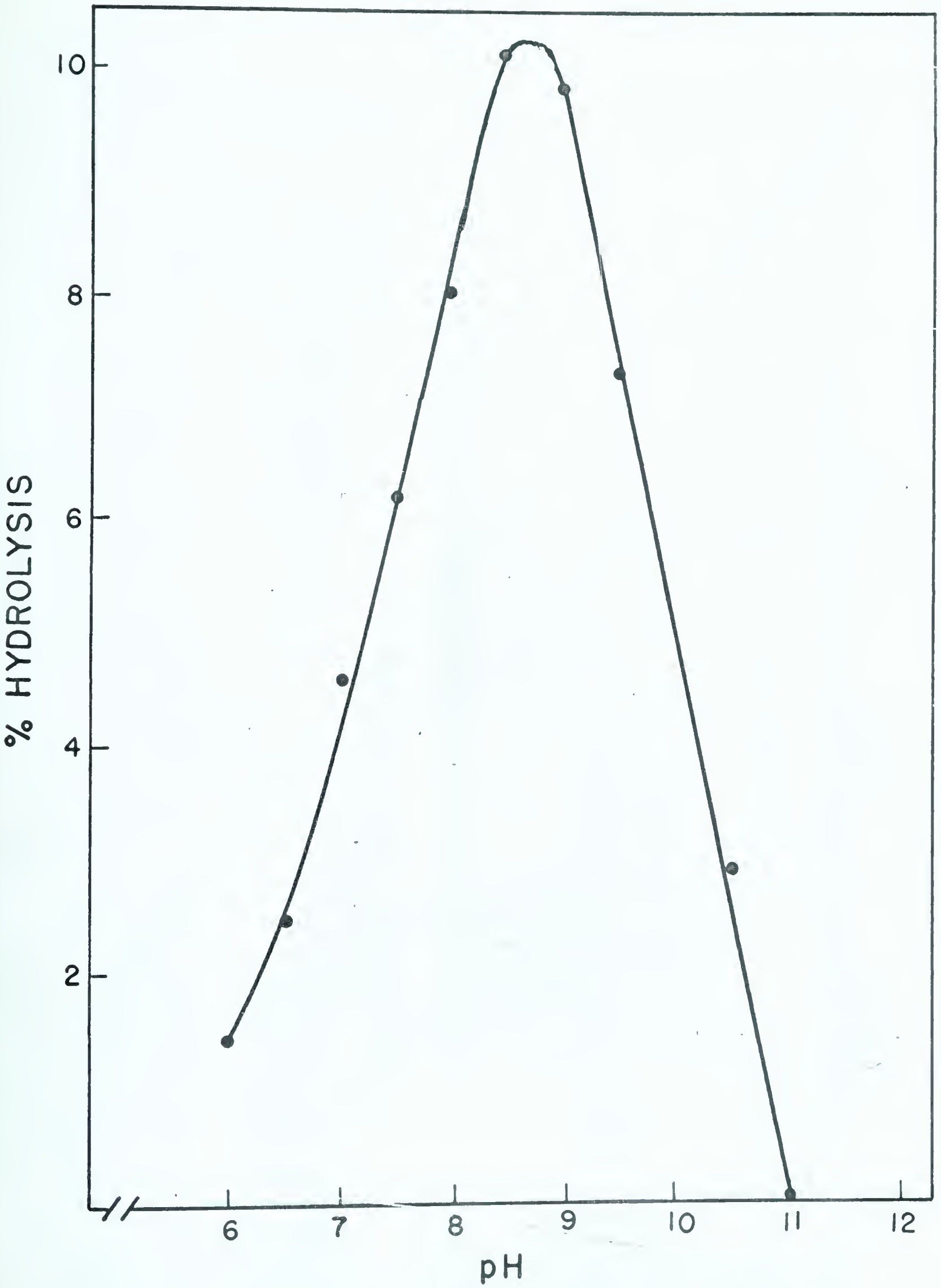


Fig. 8. INFLUENCE OF pH ON THE ACTIVITY OF PANCREATIC LIPASE WITH MILK FAT AS SUBSTRATE

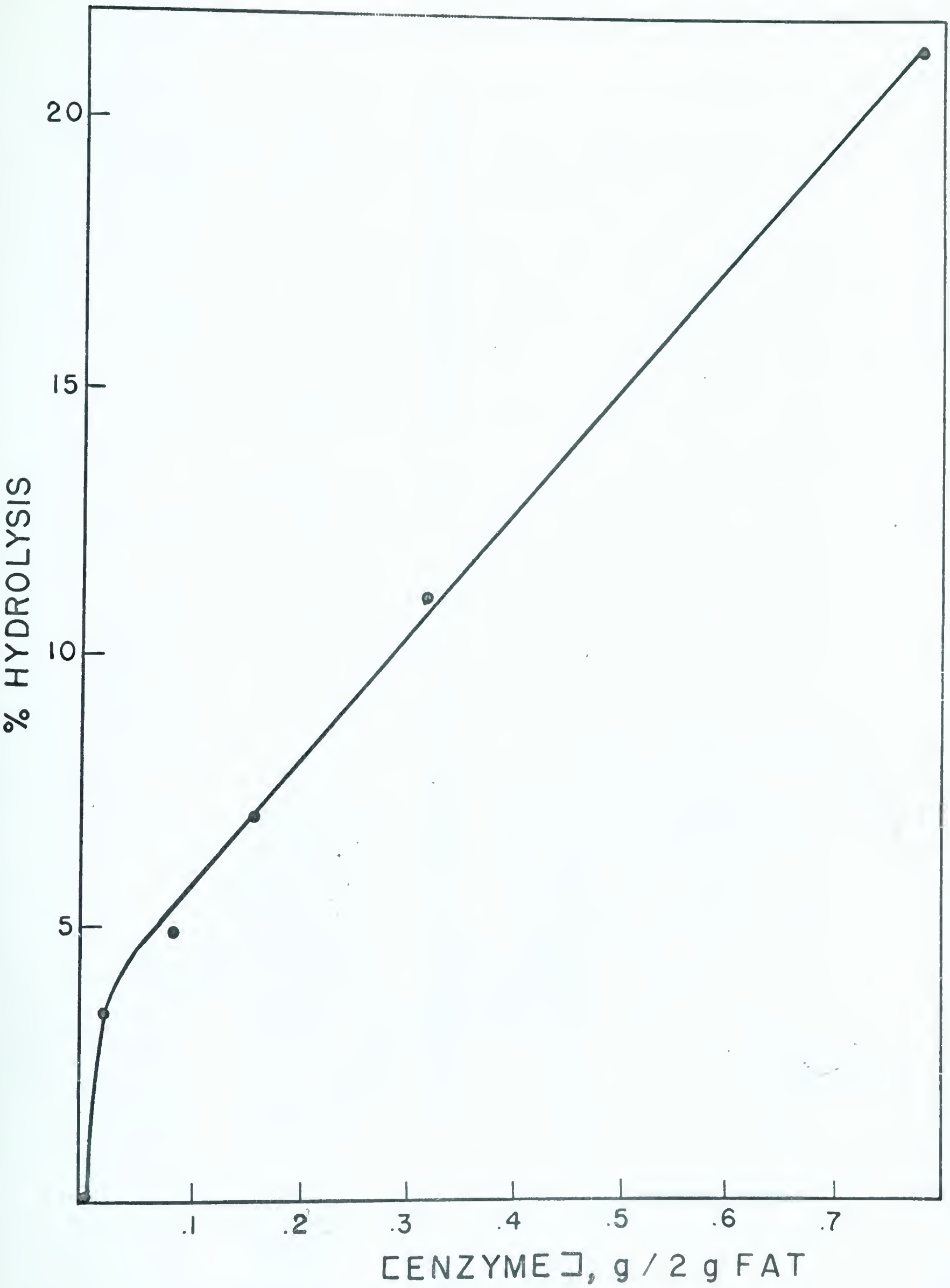


Fig. 9. EFFECT OF CONCENTRATION OF PANCREATIC LIPASE ON THE DIGESTION RATE OF MILK FAT

mum value (Vm). In this respect, the results may be considered as a Michaelis curve. As already mentioned under the heading of "Effect of enzyme concentration", no information is available to explain the non linear characteristic of the curve which has been observed by some other investigators (89).

However, such a property was not shown by esterases (37, 41, 42, 89). When purified liver esterase was assayed against increasing substrate concentration, a typical Michaelis curve was obtained. This liver esterase and pancreatic lipase have different specificities with regard to the physical state of their respective substrates. Liver esterase acts, as most enzymes do, in true solutions together with its substrate. On the other hand, pancreatic lipase (28, 89), a very peculiar enzyme indeed, can act only on esters present in a state of insolubility. Therefore, the "apparent" Michaelis curve obtained for milk fat could be partly explained as follows (Figure 10)(89). When practically no interface exists i.e. at low substrate concentration, pancreatic lipase would be dissolved in water but rather inactive. In

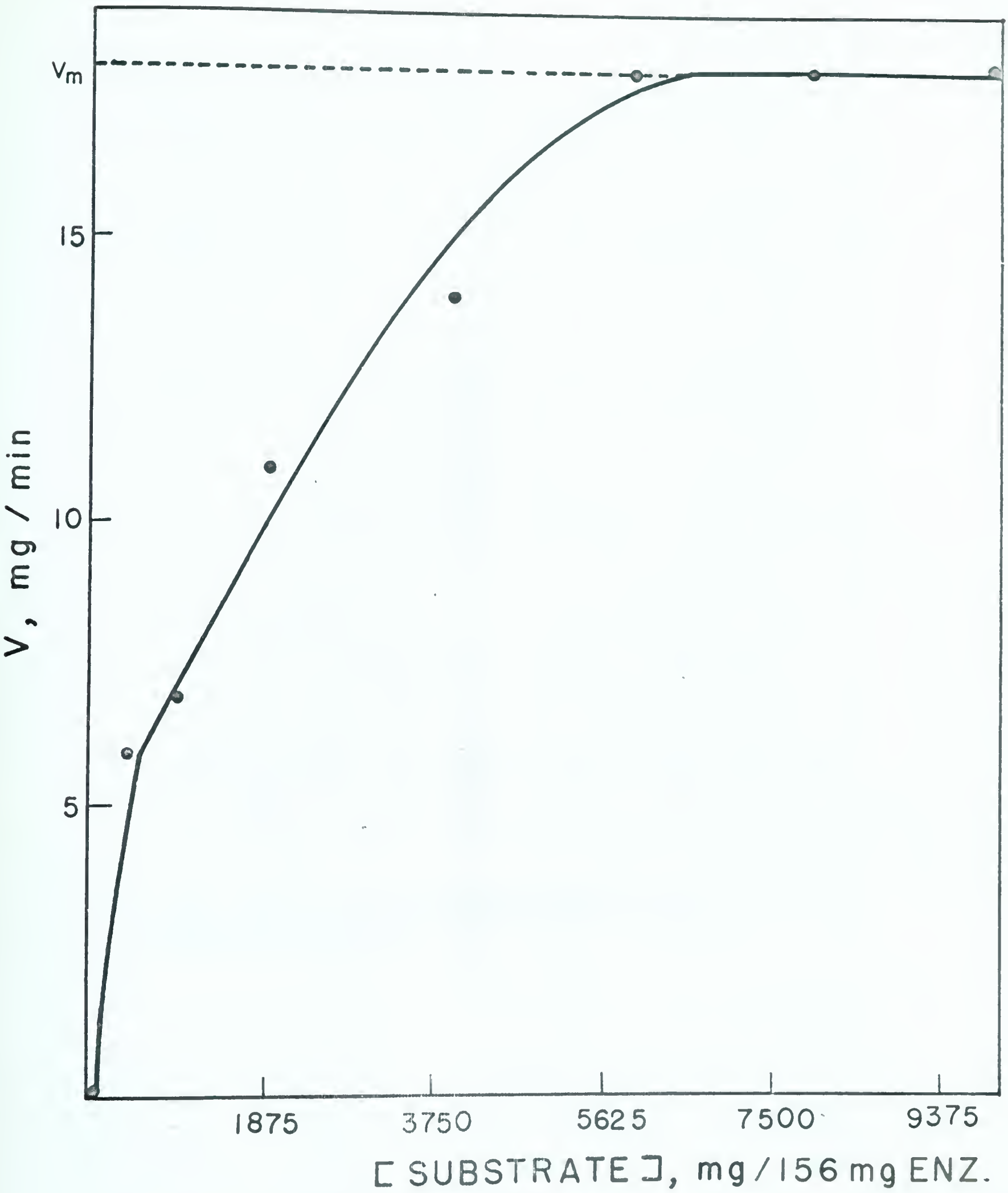


Fig. 10. INFLUENCE OF MILK FAT CONCENTRATION ON ACTIVITY OF PANCREATIC LIPASE

the presence of a large interface, as the substrate concentration increases, the activity of pancreatic lipase would increase disproportionally which is noted by a sudden break in the first portion of the curve. Finally, with a very large amount of milk fat (5 - 6g), the curve slopes off because a state of oversaturation would be reached. In the case of pancreatic lipase the usual formation of the enzyme-substrate complex can be envisaged as the adsorption of the enzyme on the insoluble substrate which would depend on the concentration of the substrate in the reaction mixture. However, a complete interpretation of the physical state inducing lipase activity is not possible at this time (89).

2. Hydrolysis of milk fat before and after interesterification

The selective release of fatty acids from genuine and interesterified milk fat was carried out with pancreatic lipase until about 30% hydrolysis of the total ester groups. The optimum conditions of lipo-

ysis were chosen as described in the preceding sections and were essentially the same as described under the heading of "Experimental procedure". The products of digestion were separated by chromatography on Florisil with further identification of the glyceride fractions on thin layer chromatography (Table 7 and Figure 11). Some authors (2, 103) studied the distribution of fatty acids in the original triglycerides by analyzing the free fatty acids. However, appreciable amounts of glycerol may be formed in these hydrolyses (18, 44) so that some of the free fatty acids are released from the internal position. For this reason, as suggested by Coleman (17), the fatty acid composition of the various glyceride fractions obtained from both fats was estimated by gas liquid chromatography. The results are presented in Table 8. The gas liquid chromatograms of the monoglyceride fractions of both fats are given in Figure 12.

Table 7. GLYCERIDE FRACTIONS AND FREE FATTY ACIDS (FFA) OBTAINED AFTER HYDROLYSIS OF GENUINE AND INTERESTERIFIED SEPTEMBER MILK FAT BY PANCREATIC LIPASE [a,c]

Samples	per cent Hydrolysis	Weight per cent				per cent Recovery [b]
		TG	DG	MG	FFA	
Genuine	29.0	28.0	28.5	17.4	26.1	97.5
Interesterified	29.1	31.4	24.7	17.0	26.9	97.5

a. Average data of four analyses of each sample.

b. The low recovery is attributed to the glycerol content of the digestion products (44).

c. In this case, 20 mg of bile salts / 75 ml was added.

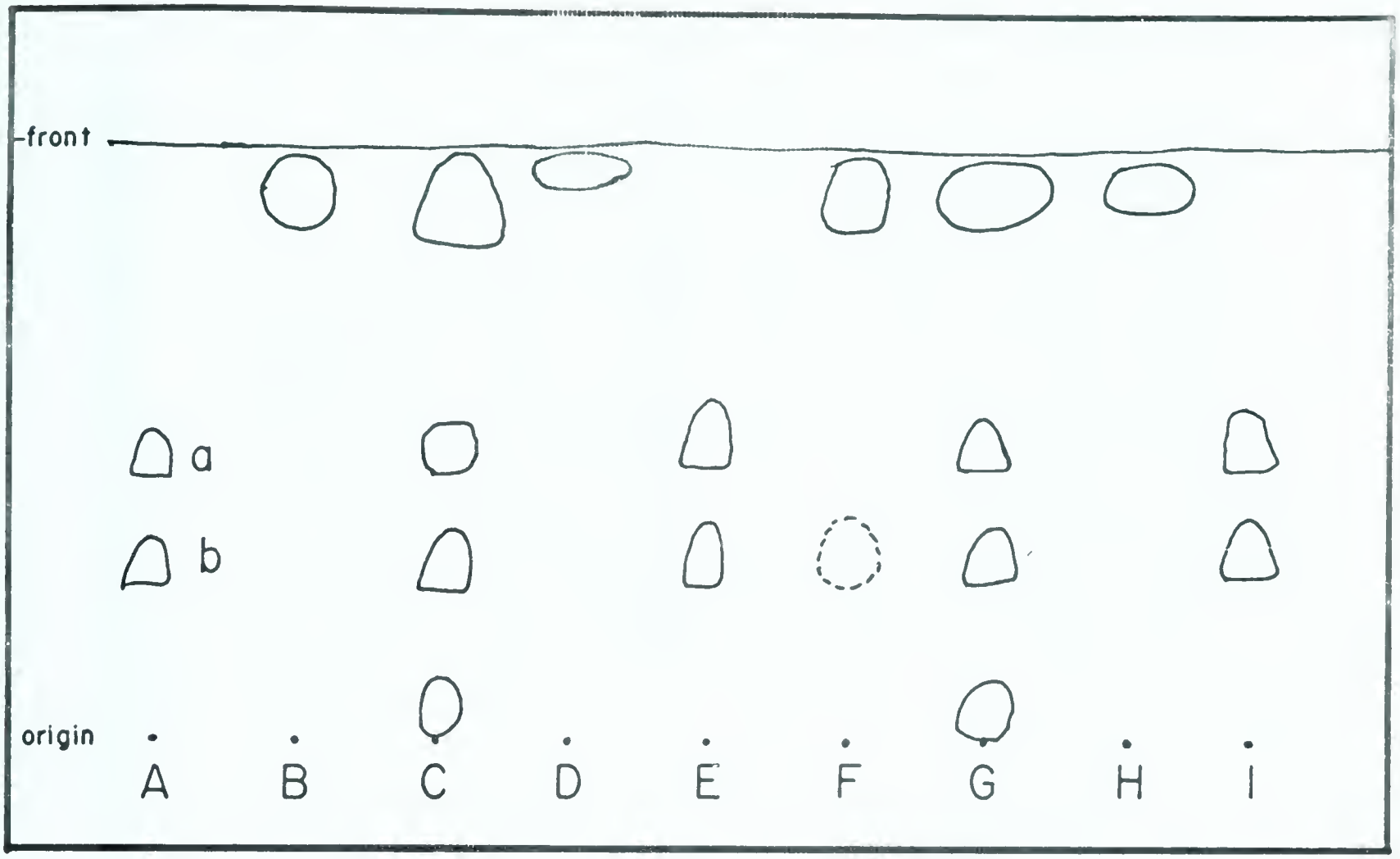


Fig. 11. THIN LAYER CHROMATOGRAM OF GENUINE AND INTERESTERIFIED SEPTEMBER MILK FAT BEFORE AND AFTER HYDROLYSIS. Samples: A. Lard diglycerides, B. Genuine milk fat before hydrolysis, C. Genuine milk fat after hydrolysis, D. Triglyceride fraction of genuine hydrolyzed milk fat obtained by column chromatography on Florisil, E. Diglyceride fraction, F. Interesterified milk fat before hydrolysis, G. Interesterified milk fat after hydrolysis, H. Triglyceride fraction, I. Diglyceride fraction. Sample size: 10 µl of 1% solution in petroleum ether. Mobile phase: stationary phase and spot detection as described in "Experimental procedure". Further details: A dashed area indicates a weak spot. From origin to front: Monoglycerides and free fatty acids; Diglycerides: (a) 1,3-diglyceride, (b) 1,2-diglyceride; Triglycerides.

Table 8. FATTY ACID COMPOSITION AS WEIGHT % METHYL ESTER OF GENUINE AND INTERESTERIFIED SEPTEMBER MILK FAT AND THE PARTIAL GLYCERIDES OBTAINED AFTER ABOUT 30% HYDROLYSIS OF BOTH FATS BY PANCREATIC LIPASE

Fatty Acid	Genuine September milk fat				Interesterified September milk fat			
	Original	after hydrolysis TG	DG	MG	Original	after hydrolysis TG	DG	MG
4:0	2.5	1.7	2.1	0.1	2.5	1.6	1.0	0.3
6:0	1.5	1.3	1.6	0.2	1.5	1.3	1.0	0.8
8:0	1.0	0.6	0.7	0.5	1.0	0.6	0.5	0.8
10:0	2.1	1.8	2.0	2.5	2.1	1.6	1.5	2.6
10:1	0.3	0.2	0.2	0.2	0.3	0.2	0.3	0.2
12:0	2.8	2.4	3.1	4.2	2.8	2.1	2.3	3.2
14:0	10.1	9.3	12.0	18.6	10.0	9.6	9.7	12.5
14:1	2.8	2.8	2.7	4.2	3.0	3.5	2.5	2.7
15:0 + 16:0 iso	2.1	2.3	1.8	2.8	2.5	2.6	2.0	2.3
16:0	26.7	26.0	30.6	31.5	26.9	26.7	28.0	29.0
16:1	3.9	4.1	4.4	4.6	4.1	3.4	3.5	3.7
17:0 + 17:0 iso	1.1	1.3	1.3	0.9	1.1	2.2	1.2	1.2
18:0	12.6	13.3	11.8	6.8	12.3	12.1	15.0	12.4
18:1	26.0	29.2	23.8	19.7	26.0	27.5	26.7	24.8
unidentified (20)	[a]	-	-	-	-	0.8	0.9	-
18:2	1.7	1.9	1.1	1.8	1.5	2.0	2.0	1.6
18:3	2.8	1.8	0.8	1.4	2.4	2.2	1.9	1.9

a. A dash means that the methyl ester is negligible on the gas chromatograms.

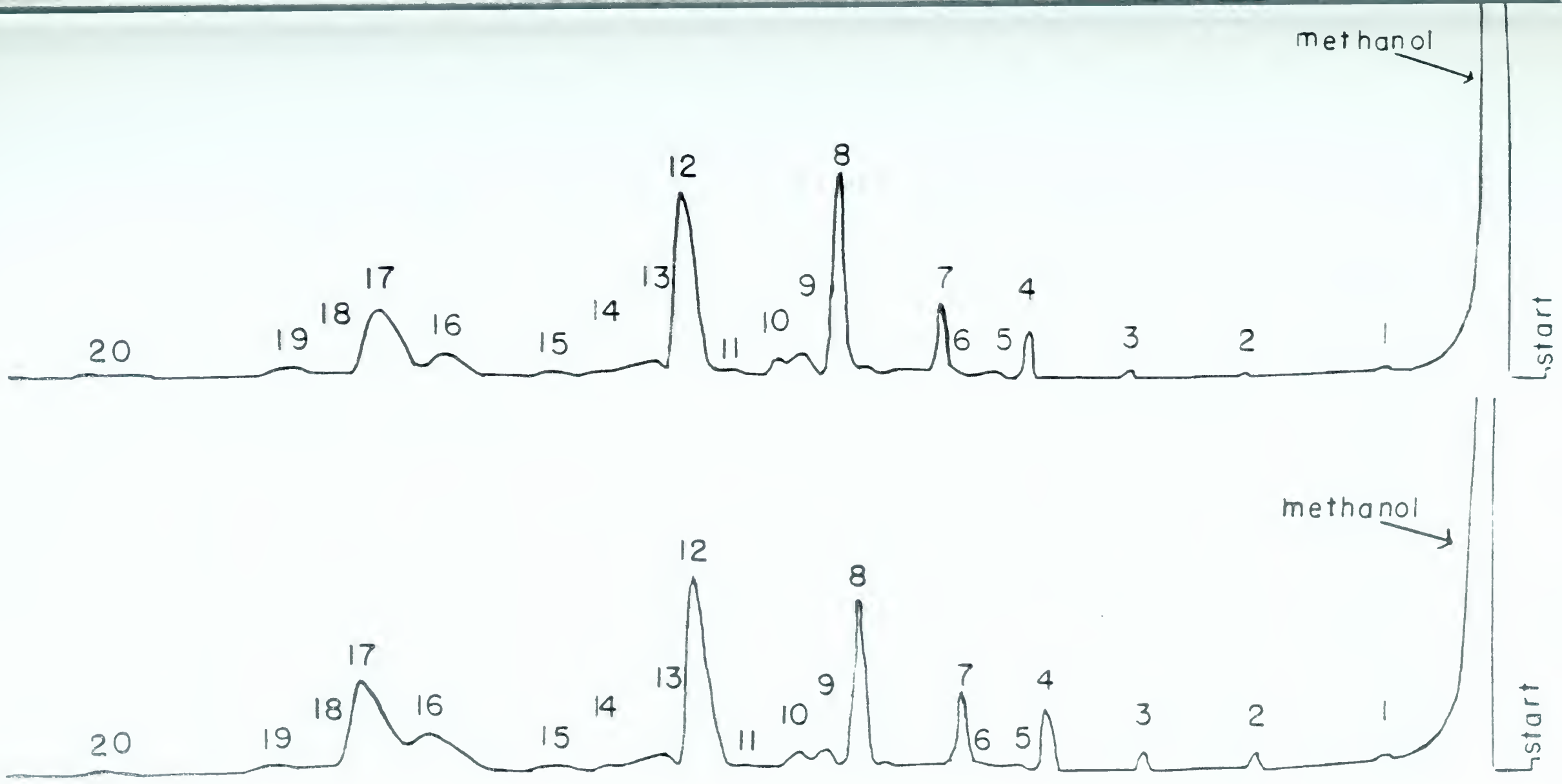


Fig. 12. GAS CHROMATOGRAMS OF METHYL ESTERS OF MONOGLYCERIDE FRACTIONS OF GENUINE (UPPER) AND INTERESTERIFIED (LOWER) SEPTEMBER MILK FAT OBTAINED AFTER ABOUT 30% HYDROLYSIS BY PANCREATIC LIPASE.
Peaks in order of appearance: (1) 4:0, (2) 6:0, (3) 8:0, (4) 10:0, (5) 10:1, (6) 11:0, (7) 12:0, (8) 14:0, (9) 14:1, (10) 15:0, (11) 16:0 ^{iso}, (12) 16:0, (13) 16:1, (14) 17:0, (15) 17:0^{iso}, (16) 18:0, (17) 18:1, (18) unidentified, (19) 17:2, (20) 18:3.

E. THE CONTENT OF MILK FAT GLYCERIDES AND THE FATTY ACID COMPOSITION OF MILK FAT GLYCERIDES COMPARED WITH THAT OF PARTIAL GLYCERIDES OBTAINED BY PANCREATIC LIPASE HYDROLYSIS

Four samples of milk fat were designated as follows: (1) cow No.4, January 29th, 1962; (2) cow No.2, January 29th, 1962; (3) bulk tank, January 29th, 1962; (4) October, 1963. Each sample was separated into glyceride classes by column chromatography on Florisil followed by identification of each fraction by thin layer chromatography as described under the heading "Experimental procedure". The diglyceride and triglyceride fractions, obtained by chromatography on Florisil, together with the original milk fat were methylated and analyzed by dual column, temperature programmed gas liquid chromatography.

Many factors are known to influence milk fat composition. Thus, it is impossible to characterize milk fat by an exact amount of any individual constituent. Estimates of lipid components are best expressed as ranges of values.

The results of Florisil chromatography (Table 9)

indicate the relative amounts of each glyceride class in these fats. Reproducibility of the column chromatographic separation was excellent. The results listed in Tables 10 & 11 for samples (1) and (4) were the average of 7 and 15 replicates respectively, performed to obtain sufficient material for gas liquid chromatographic analysis of the fatty acid composition.

Table 9. TRI-, DI- AND MONOGLYCERIDE CONTENT OF INDIVIDUAL AND MIXED MILK FATS AS DETERMINED BY COLUMN CHROMATOGRAPHY ON FLORISIL

Sample No.	Weight % TG	DG	MG	Recovery %
1. Individual	94.4	5.5	0.1	100.2
2. Individual	93.1	6.6	0.3	100.4
3. Mixed	93.4	6.4	0.2	99.4
4. Mixed	95.4	4.4	0.2	100.0

Table 10. TRI-, DI- AND MONOGLYCERIDE CONTENT OF SEVEN REPLICATES OF INDIVIDUAL MILK FAT, SAMPLE NO. 1., AS DETERMINED BY COLUMN CHROMATOGRAPHY ON FLORISIL

Replicate No.	Weight % TG	DG	MG	Recovery %
1	94.6	5.4	0.0	100.4
2	94.9	5.0	0.1	100.9
3	94.7	5.1	0.2	99.4
4	94.3	5.6	0.1	100.5
5	93.1	6.6	0.3	100.1
6	94.9	5.0	0.1	100.2
7	94.1	5.7	0.2	100.2
Average	94.4	5.5	0.1	100.2

Table 11. TRI-, DI- AND MONOGLYCERIDE CONTENT OF FIFTEEN REPLICATES OF MIXED MILK FAT, SAMPLE NO.4, AS DETERMINED BY COLUMN CHROMATOGRAPHY ON FLORISIL

Replicate No.	Weight % TG	DG	MG	Recovery %
1	95.5	4.2	0.3	100.4
2	95.8	4.2	0.0	99.7
3	95.6	4.4	0.0	100.1
4	95.5	4.4	0.1	99.9
5	95.9	4.0	0.1	99.8
6	95.5	4.4	0.1	99.8
7	95.5	4.5	0.0	100.2
8	95.0	4.9	0.1	99.9
9	94.4	4.9	0.7	100.5
10	95.7	4.0	0.3	99.9
11	95.6	4.0	0.4	100.3
12	95.2	4.3	0.5	100.2
13	95.0	4.6	0.4	99.9
14	95.7	4.3	0.0	99.4
15	95.4	4.3	0.3	99.9
Average	95.4	4.4	0.2	100.0

The monoglyceride content of both 1962 and 1963 milk fats was very small, about 0.1 - 0.3%. These figures are in agreement with the findings of Mehlenbacher (76: p 492). In addition, substantial quantities of diglycerides were found. These data for glycerides in Table 9 show that in the milk fat samples examined, the diglycerides ranged from 4.4 - 6.6%. As the acid degree values of both fat samples were too small to account for the difference in the diglyceride content, hydrolysis during storage must be ruled out as a cause of a higher diglyceride content in the 1962 milk fat. These ranges of values of diglycerides were 5.0 - 6.6% for the 1962 milk fat and 4.0 - 4.9 for the 1963 fresh milk fat.

Although there were variations in the data for the four samples of milk fat (Table 9), the occurrence of monoglycerides and particularly diglycerides seemed characteristic. The results showed that milk fat contained 0.1 - 0.2% of monoglycerides and 4 - 6% of diglycerides. Since this work was done Mickle et al. (78) have published figures on the glycerides present in a sample of milk fat, they found 94, 5

and 1% of tri-, di- and monoglycerides respectively.

The question of whether the glyceride classes overlapped during the column chromatography was answered by the ability of thin layer chromatography to separate lipid classes. The identification of the two isomeric forms of diglycerides were made by using 1,3- and 1,2-diglycerides of fresh lard. The thin layer chromatogram (Figure 13) provided positive assurance that the glyceride classes were pure and that the natural diglycerides consisted largely of the 1,2-isomers. Similar results were obtained with all four samples of milk fat investigated.

The question of whether the diglycerides are artifacts of lipolysis in the milk lipids is an important one. The average quantities of free fatty acids (FFA), monoglycerides (MG) and diglycerides (DG) from mixed milk fat are presented in Table 12.

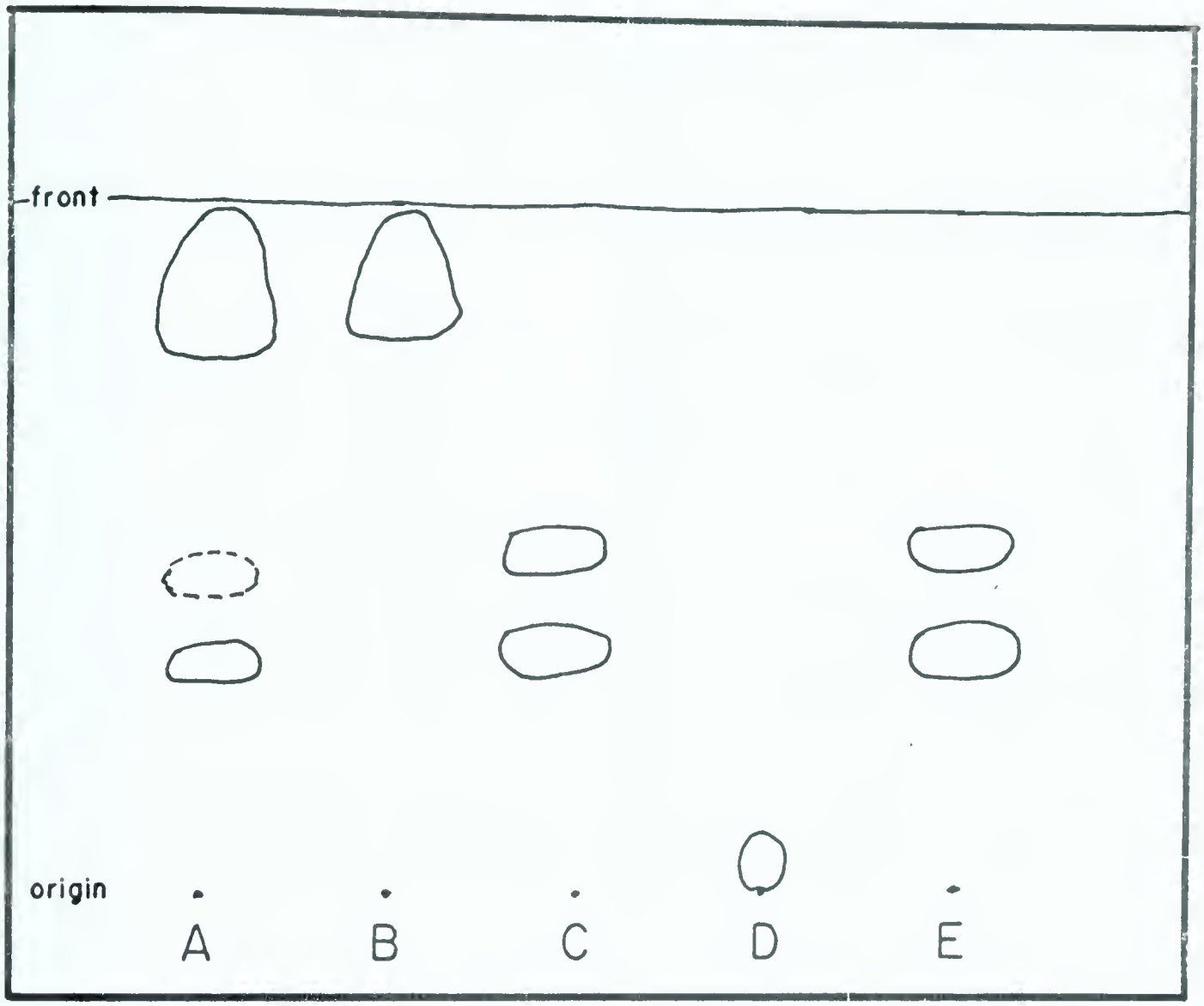


Fig. 13. THIN LAYER CHROMATOGRAM OF ORIGINAL MILK FAT, PARTIAL GLYCERIDES OF ORIGINAL MILK FAT AND LARD DIGLYCERIDES.
Samples: A.Fresh cow milk fat, B. Triglyceride fraction, C. Diglyceride fraction, D.Monoglyceride fraction, E. Lard diglycerides. Sample size: 10 µl of 1% solution in petroleum ether. Mobile phase, stationary phase and spot detection as described in "Experimental procedure". Further details: A dotted area indicates a weak spot. The slowest moving diglyceride spot is the 1,2-isomer (83).

Table 12. COMPARISON OF THE FREE FATTY ACIDS (M.W. - 244) AND PARTIAL GLYCERIDE CONTENT OF MIXED MILK FAT

	mMoles/100g fat
Monoglycerides	0.80
Diglycerides	9.00
Free fatty acids (from ADV determination)	0.75
Free fatty acids (calculated)[a]	10.60

a. FFA (calculated) = 2mM MG + mM DG = 0.8 x 2 + 9.0 = 10.60

Calculations show that the amount of diglycerides was too large to account for the quantity of recovered free fatty acids as determined from the Acid Degree Value (ADV) (77). A large number of milk fats have been analyzed in this laboratory with similar results. Moreover, the hydroxyl value (77) was estimated and was close to the theoretical value calculated from the column chromatographic results. The conclusion must be that the diglycerides are not products of hydrolysis as has been suggested in the case of monoglycerides (48). It is probable that the diglycerides are left over

from fat synthesis in the mammary gland.

The original fats and their diglycerides were converted to methyl esters and analyzed by gas liquid chromatography. Results of these analyses are listed in Table 13. A typical gas chromatogram of the natural diglycerides of milk fat is reproduced in Figure 14.

The fatty acid composition of the triglycerides was similar to that of the original fat. The fatty acid composition of the natural diglycerides and triglycerides showed that butyric (4:0), caproic (6:0), stearic (18:0) and oleic (18:1) acids were found in lower concentrations in the natural diglycerides; on the other hand, palmitic acid (16:0) was the only acid found in significantly higher concentration in the diglycerides. Similar results were found with the individual and mixed milk fats.

Table 13. FATTY ACID COMPOSITION AS WEIGHT % METHYL ESTER OF TWO MILK FATS, THE TRI- AND DIGLYCERIDES SEPARATED BY COLUMN CHROMATOGRAPHY ON FLORISIL AND THE PARTIAL GLYCERIDES OBTAINED AFTER ABOUT 30% HYDROLYSIS OF THE FATS BY PANCREATIC LIPASE

Fatty acid	Individual milk fat, sample No.1						Mixed milk fat, sample No. 4					
	Original			After hydrolysis			Original			After hydrolysis		
	whole fat	TG	DG	TG	DG	MG	whole fat	TG	DG	TG	DG	MG
4:0	3.6	3.4	2.7	2.5	2.6	0.2	2.4	2.3	1.3	1.9	2.0	0.1
6:0	1.8	1.9	1.3	1.5	1.9	0.3	1.4	1.4	0.8	1.3	1.4	0.2
8:0	1.0	0.9	0.9	0.8	0.8	1.0	0.9	0.8	0.6	0.7	0.9	0.6
10:0	1.9	2.0	2.0	1.6	2.2	2.8	2.1	1.9	2.1	1.6	2.6	2.7
10:1+11:0[a]	0.4	0.3	1.3	0.4	0.3	0.3	0.3	0.2	0.8	0.2	0.2	0.2
12:0	2.5	2.6	2.5	2.3	2.5	3.9	2.5	2.7	2.5	2.3	2.9	3.7
14:0	10.1	10.0	10.7	9.4	12.1	19.5	9.6	9.3	9.5	9.3	11.4	15.9
14:1	2.5	2.0	2.3	2.2	2.9	3.6	1.8	2.1	2.3	2.4	2.9	3.5
15:0+16:0 iso	2.0	1.9	1.7	1.5	1.8	1.6	1.7	1.9	1.8	1.8	2.0	2.2
16:0	31.4	30.7	32.9	31.5	35.4	32.7	25.9	24.1	29.3	24.8	27.4	29.9
16:1	2.3	1.9	2.0	2.5	2.6	4.1	2.2	1.9	1.9	2.1	2.0	2.8
17:0+17:0 iso	2.3	2.0	2.5	2.3	2.0	1.9	2.0	2.8	2.8	3.8	3.4	4.6
18:0	8.5	9.9	8.9	9.9	8.4	4.2	14.4	15.6	13.3	14.7	11.9	8.3
18:1	26.5	27.4	25.0	27.8	21.4	20.5	27.7	27.4	25.8	28.5	24.8	21.4
unidentified (20)	1.2	1.1	0.8	1.1	1.0	0.8	0.9	1.2	1.0	1.0	0.9	1.0
18:2	1.2	1.2	1.3	1.7	1.3	1.7	1.8	2.4	2.1	2.0	1.7	1.9
18:3	1.0	0.8	1.2	1.0	0.8	0.9	2.4	2.0	2.1	1.6	1.6	1.0

a. The peak of this fatty acid is not normally discernible on chromatograms of whole milk fat, but does show upon chromatograms of some of the partial glycerides.

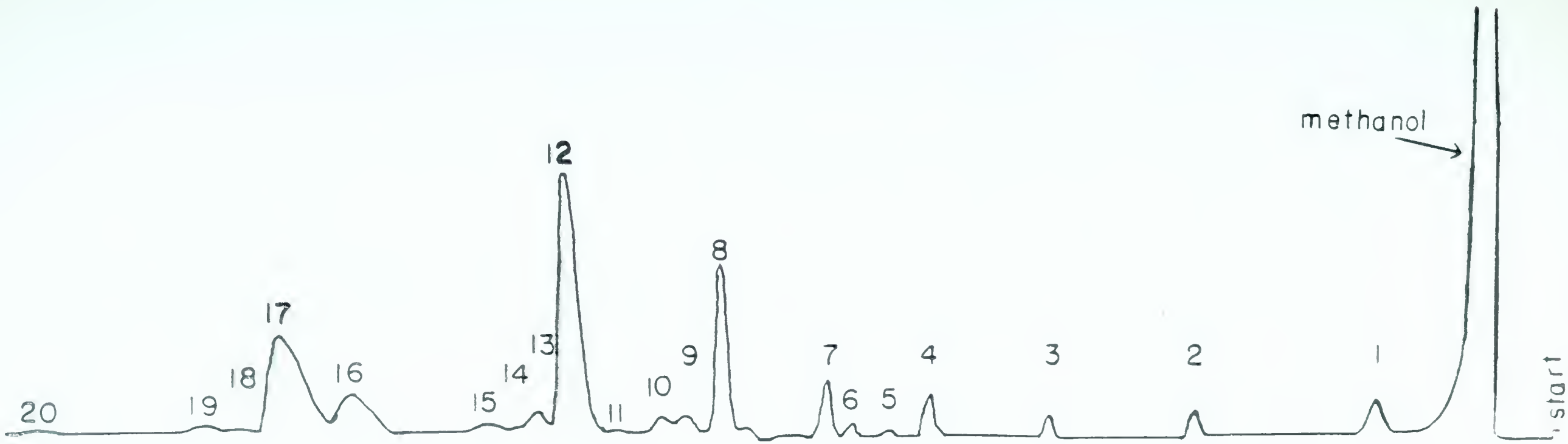


Fig. 14. GAS CHROMATOGRAM OF METHYL ESTERS FROM THE NATURAL DIGLYCERIDE FRACTION OBTAINED FROM COLUMN CHROMATOGRAPHY ON FLORISIL.
Peaks in order of appearance: (1) 4:0, (2) 6:0, (3) 8:0, (4) 10:0, (5)10:1, (6) 11:0, (7) 12:0, (8) 14:0, (9) 14:1, (10) 15:0, (11) 16:0 ^{iso}, (12) 16:0, (13) 16:1, (14) 17:0, (15) 17:0^{iso}, (16) 18:0, (17) 18:1, (18)unidentified, (19) 17:2, (20) 18:3.

Therefore, it was interesting to compare the fatty acid composition of natural milk fat diglycerides with that of partial glycerides obtained by the action of pancreatic lipase on these milk fats.

The hydrolysis was stopped after about 30% hydrolysis of the total ester groups present. Then, the digestion products were separated into glyceride fractions by Florisil chromatography and identified by thin layer chromatography. Finally, each glyceride fraction was analyzed by dual column, temperature programmed gas liquid chromatography (Table 13). The results of the column chromatography are given in Tables 14 & 15. The thin layer chromatogram (Figure 15) illustrates the sharpness of separation of each glyceride group.

Table 14. FLORISIL CHROMATOGRAPHY OF NEUTRAL GLYCERIDES, EXPRESSED AS WEIGHT %, AFTER ABOUT 30% HYDROLYSIS OF INDIVIDUAL MILK FAT BY PANCREATIC LIPASE[a]

Replicate No.	Sample No.1			Recovery
	TG	DG	MG	%
1	58.5	27.7	13.8	96.4
2	57.2	28.8	14.0	99.7
3	58.4	28.3	13.3	96.7
4	58.7	27.3	14.0	96.5
5	59.1	27.1	13.8	96.4
6	60.0	26.2	13.8	96.3
7	59.6	26.4	14.0	98.8
8	59.4	26.7	13.9	98.0
9	58.6	27.7	13.7	94.4
10	58.9	27.3	13.8	99.0
Average	58.8	27.2	13.8	97.2

a. In this case, 5 mg of bile salts / 75 ml was added.

Table 15. FLORISIL CHROMATOGRAPHY OF ACIDIC GLYCERIDES, EXPRESSED AS WEIGHT %, AFTER ABOUT 30% HYDROLYSIS OF MIXED MILK FAT BY PANCREATIC LIPASE.[b]

Replicate No.	Sample No.4				Recovery %
	TG	DG	MG	FFA	
1	27.1	29.5	18.4	25.0	96.5
2	27.0	29.8	18.6	24.6	96.6
3	27.1	29.1	18.3	25.5	97.2
4	27.1	29.5	18.4	25.0	98.1
Average	27.1	29.5	18.4	25.0	97.1[a]

a. The low recovery is attributed to the glycerol content of the digestion products (44).

b. In this case, 20 mg of bile salts / 75 ml was added.

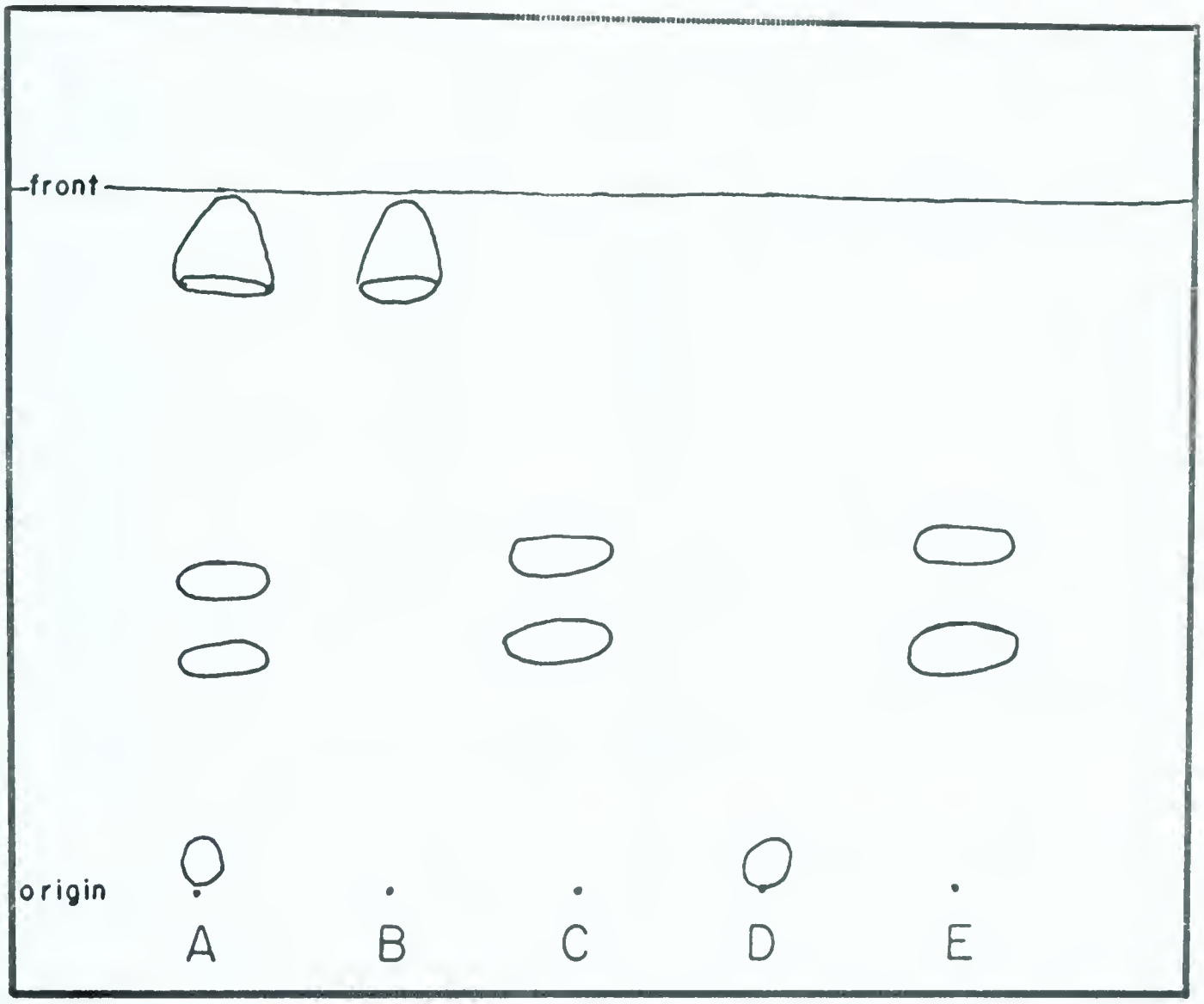


Fig. 15. THIN LAYER CHROMATOGRAM OF LARD DIGLYCERIDES AND MILK FAT AFTER ABOUT 30% HYDROLYSIS BY PANCREATIC LIPASE.
Samples: A. Hydrolyzed milk fat, B.Triglyceride fraction of hydrolyzed milk fat, C. Diglyceride fraction, D.Monoglyceride fraction, E.Lard diglycerides. Sample size: 10 µl of 1% solution in petroleum ether. Mobile phase, stationary phase and spot detection as described in "Experimental procedure". Further details: From origin to front: Monoglycerides or/and free fatty acids, diglycerides, triglycerides. The slowest moving diglyceride spot is the 1,2-isomer (83).

Methyl ester analysis of the natural and hydrolyzed diglycerides indicated that they were similar in composition.

The fatty acid composition of the natural triglycerides of milk fat and that of the residual triglycerides was similar for the long chain fatty acids but there was no good agreement for the short chain ones, particularly butyric acid. Thus, it did appear that pancreatic lipase preferentially released butyric acid from the original triglycerides of milk fat.

The fatty acid composition of the original fat and the di- and monoglycerides resulting from the enzymatic hydrolysis were as could be expected i.e. methyl esters which were low in the residual triglyceride fraction were higher in the residual diglycerides and highest in the residual monoglycerides.

The residual monoglycerides were somewhat enriched in the saturated acids, capric to palmitic (10:0 - 16:0) and particularly in myristic acid (14:0). They were poorer in the unsaturated acids, and in these respects, milk fat tended to resemble the unique distribution of fatty acids observed for pig fat (71). These results

agreed well with those of McCarthy *et al*. (75) and Patton *et al*. (82). In all of these studies pancreatic lipase was used to digest milk fat.

DISCUSSION

The compararive rates of hydrolysis of different triglycerides indicatee that pancreatic lipase hydrolyzed short chain fatty acid glycerides more rapidly tha milk fat or any other triglyceride. Trilaurin, trimyristin and triolein were attacked slowly. Therefore, the enzyme was relatively more active on the short chain fatty acid triglycerides than on the long chain and unsaturated fatty acid ones (Table 2). This apparent selectivity of the enzyme supports the belief that pancreatic lipase shows specificity for short chain fatty acids. Because of the extreme difficulty in preparation of comparable emulsions with two triglycerides differing widely in molecular weight, melting point and solubility, the validity of these comparisons might be open to question.

It was therefore interesting to know whether the pattern of stepwise hydrolysis of short chain fatty acid triglycerides is the same as when pancreatic lipase acts on long chain triglycerides.

The hydrolysis patterns of a long chain fatty acid triglyceride, 2-oleoyl-dipalmitin (67) and a

short chain fatty acid one, tricaprylin, are shown in Figures 3 & 4. The glyceride curves of both synthetic fats indicated a series of consecutive reactions. The concentration of unhydrolyzed triglycerides decreased sharply; the concentration of diglycerides rose rapidly to a maximum and decreased slowly for the rest of the digestion period. During the initial period of hydrolysis, the rate of formation of monoglycerides was rather slow, then increased as soon as the rate of formation of diglycerides reached a maximum. The only possible explanation of these observations is to consider the enzymatic digestion of both glycerides as a directed stepwise hydrolysis; thus:

Triglyceride $\longrightarrow$ Diglyceride $\longrightarrow$ Monoglyceride

The hydrolysis of tricaprylin presented some interesting facts. In the same period of time (10 min) and under similar conditions of digestion, a larger amount of partial glycerides together with a smaller amount of unhydrolyzed triglycerides were obtained from the hydrolysis of tricaprylin. In addition, approximately 10% (Table 5) of the combined glycerol has been hydrolyzed

to free glycerol whereas this occurs only at the end of a 90 minute hydrolysis period with the long chain fatty acid triglyceride as the original substrate. Consequently, the hydrolysis of a short chain fatty acid glyceride seems more complete than that of a long chain fatty acid one. It was also observed that the proportions of 1-isomer in the monoglyceride fraction of tricaprylin was found to be as high as 50% (Table 5) in comparison with about 25% in the monoglyceride fraction of 2-oleoyl-dipalmitin (67).

This means that contrary to longer chains, the caprylyl chain attached to the internal position in the tricaprylin molecule is rapidly hydrolyzed. The easy release of a short chain fatty acid can be explained either by a preference of pancreatic lipase for short chain fatty acids or by a higher rate of isomerization of 1,2-diglycerides and 2-monoglycerides containing short chain fatty acids.

The possible relation between positional specificity of pancreatic lipase and chain length specificity was also studied by hydrolyzing an equimolar mixture of a short and long chain fatty acids such

as tricaprylin-triolein.

Gas chromatography of the methyl esters of fatty acids in partial glycerides, obtained after various degrees of hydrolysis showed that pancreatic lipase preferentially liberated caprylic acid rather than oleic acid. Approximately two moles of caprylic acid were released after 15% hydrolysis of the total ester groups present and more than three moles after 30% of total hydrolysis (Table 7).

These data provided a basis for a sound explanation for the selective release of short chain fatty acids by pancreatic lipase. As pancreatic lipase hydrolyzed the short chain fatty acids more rapidly and more completely than the long ones, the internal position of a short chain fatty acid triglyceride is significantly hydrolyzed so that the chain length specificity of the enzyme takes preference over the position specificity.

Fats are not mixtures of simple triglycerides but rather contain molecules of the mixed type comprising two or three different fatty acids (81). The study of chain length specificity of pancreatic lipase is better approached by investigating a natural fat in which the specific fatty acid distribution was completely randomized. The effects of the positional specificity are eliminated because different fatty acids occupy equivalent position.

In the case of natural fats consisting of short chain fatty acids, the question of whether there is any selective hydrolysis of particular triglycerides was answered by subjecting to hydrolysis milk fat samples before and after interesterification (randomization).

If a natural fat consisting of long chain fatty acids was subjected to hydrolysis before and after interesterification, we would expect the same fatty acid composition in each glyceride fraction because long chain fatty acids are removed at about the same rate (16, 68).

When genuine and interesterified milk fat was

hydrolyzed, the original fatty acid composition together with the residual triglycerides of both fats followed the same pattern. The triglyceride fractions of both fats showed a preferential release of short chain fatty acids (butyric, caproic, caprylic and capric acids). In the case of interesterified milk fat, a progressive decrease of short chain fatty acids without appreciable change of long ones was noticed in the di- and monoglyceride fractions. This indicated that long and short chain fatty acids were split off at different rates. On the other hand, the fatty acid composition of mono- and diglycerides resulting from the hydrolysis of genuine milk fat differed markedly from the ones of interesterified milk fat. Indeed, myristic, palmitic,stearic and oleic acids do not follow the same trend in both fats. This difference in the fatty acid composition showed that the distribution of fatty acids within the glycerides of milk fat is specific.

These results indicated that short chain fatty acids were released preferentially over long chain

fatty acids and also that they would be released from the internal position before complete release of long chain fatty acids from the external positions.

The findings of this study have considerable significance. They establish that chain length specificity of pancreatic lipase is an important factor in the investigation of the glyceridic structure of natural fats which have a high content of short chain fatty acids.

As a result of this evidence, the conclusions drawn from previous work have to be reconsidered. Recently, Jack et al. (44) have listed the requirements, which when fulfilled, would make the pancreatic lipase technique applicable to milk fat. They did not consider the possibility that short chain fatty acids may be released from the internal position before long chain fatty acids from the external positions. The results obtained by some investigators (15, 47, 50), especially Kumar et al. (56, 57) indicating that butyric acid of milk fat glycerides is located predominently in the external positions

must be reconsidered carefully.

In the course of work on the composition of milk fat evidence was obtained of the presence of diglycerides in fresh milk fat. The slowest moving spot of the 1,2-diglycerides was much stronger than the 1,3-diglyceride spot, indicating the natural diglycerides of milk fat to be predominantly the 1,2-isomer (Figure 13). It was possible that the presence of the 1,3-diglycerides was the result of isomerization during the chromatographic preparation, as was indicatee by the thin layer chromatography results. The determination of the acid degree and hydroxyl values provided evidence that the natural diglycerides are not artifacts of lipolysis.

It is probable that the diglycerides of fresh milk fat were left over from incomplete triglyceride synthesis in the mammary gland. Patton and McCarthy (83) have recently suggested that diglycerides are intermediates in milk fat synthesis and they have shown that 1,2-diglycerides are constituents of the lactating mammary gland. It was also suggested that milk fat triglycerides resulted from addition

of a short chain fatty acid to a diglyceride containing long chain fatty acid, and this led to the particular fatty acid distribution in the glycerides which has been reported by several workers (2, 15, 56, 57, 82).

It was therefore interesting to determine the fatty acid composition of the diglycerides and compare this with the results obtained with partial glycerides resulting from pancreatic lipase hydrolysis of these samples of milk fat.

The gas liquid chromatographic results afforded an opportunity to discuss the role of short chain fatty acids, particularly butyric acid in the structure and synthesis of milk fat (Table 13). The natural glycerides were lower in butyric and caproic acids than were the whole fats or triglycerides and in this respect resembled the diglycerides of mammary tissue isolated by Patton and McCarthy (83). However, levels of these short chain fatty acids were higher than found by these workers in mammary tissue lipids and this would not fit in well with the theory of formation

of milk fat triglycerides proposed by these authors.

The diglycerides obtained by pancreatic lipase hydrolysis had a somewhat higher content of short chain fatty acids than the natural diglycerides and were higher in myristic (14:0) and lower in stearic (18:0) and oleic (18:1) acids. In general, however, the composition of the two kinds of diglycerides was strikingly similar and suggested that the diglycerides were formed in the synthesizing cells of the mammary gland.

The analysis of the residual tri-, di- and monoglycerides was of interest in relation to the study of the glyceride structure of milk fat. The use of the pancreatic lipase technique for the study of glyceride structure of fats containing short chain fatty acids has been questioned (31). There was the possibility that pancreatic lipase might show a chain length specificity which might take preference over the position specificity. As already mentioned with respect to the investigation of genuine and interesterified milk fat, the same reasoning applies here. A similar pattern of fatty acid composition was ob-

tained with the residual glycerides from the pancreatic lipase hydrolysis of September milk fat as well as with individual and mixed milk fats and this confirmed the reproducibility of the results. Once again a comparison of the composition of the residual triglycerides after hydrolysis with the wholefat (Tables 8 and 13) showed a striking difference in the content of short chain fatty acids. This indicated that there is a preferential attack on a particular glyceride class. Although the figures given by Jack et al. (44) showed the same trend, they concluded that the difference was not great enough to indicate preferential hydrolysis. The trend in these results was unmistakable and included all the fatty acids from butyric to capric (from 4:0 to 10:0). There was only a small difference between the residual tri-and diglycerides with respect to the short chain fatty acids. This has been a very difficult subject to study because of difficulties in analyzing mixtures containing short chain fatty acids. (54). The method of temperature programmed gas liquid chromatography which was used, precluded losses of the volatile short chain fatty acids (20). It was possible

that the initial part of pancreatic lipase hydrolysis consists of a preferential attack on glycerides containing two short chain fatty acids of which one is released. It is to be noted from Tables 6 and 13 that the residual diglycerides after hydrolysis still contained an appreciable amount of short chain fatty acids. This suggested that the short chain fatty acids were not located largely at the external positions in the glyceride molecules of milk fat as suggested by some workers (2, 56, 57, 82). The very low content of short chain fatty acids, especially butyric (4:0), indicated that the residual monoglycerides of these acids were particularly rapidly hydrolyzed by pancreatic lipase. The following hypothesis can be suggested to explain these results: milk fat triglycerides containing butyric acid in the external positions are preferentially attacked to form 1,2-diglycerides; triglycerides containing butyric acid in the internal position yield 1,2-diglycerides with butyric acid in the internal position, which upon further hydrolysis or possibly isomerization will yield either a monoglyceride of butyric acid which is extremely unstable and rapidly hydrolyzed or have the butyric acid split off to yield a 1-monoglyceride or a long chain fatty acid.

A preferential hydrolysis of short chain fatty acids from glycerides by pancreatic lipase would produce monoglycerides that were not truly representative of the original triglycerides of milk fat as is normally the case for other fats.

In the case of milk fat and other fats containing short chain fatty acids, the results given by the pancreatic lipase hydrolysis technique reported in the literature are probably erroneous. Short chain fatty acids originally attached to the internal position may appear in the free fatty acid fraction whereas long chain fatty acids originally attached to the external positions may appear in the 2-monoglyceride fraction.

It is evident from these results that the pancreatic lipase technique cannot be used for fats that contain short chain fatty acids without a careful consideration of the possibility of a preferential hydrolysis of these acids.

The results of the long chain fatty acid content of the partial glycerides after hydrolysis of milk fat (Tables 8 and 13) were in general agreement with previous

findings (2, 44, 75, 82). The contents of saturated fatty acids, capric (10:0), lauric (12:0), myristic (14:0) and palmitic (16:0) were increased in the partial glycerides indicating their major location at the internal position of milk fat triglycerides. The 18 carbon atom fatty acids, both stearic (18:0) and oleic (18:1) showed the reverse trend, indicating their major location at the external positions. The different positional attachment of stearic acid as compared with the short chain saturated fatty acids is an unusual feature of the glyceride structure of milk fat as compared with that of other natural fats (74).

CONCLUSIONS

It was demonstrated that short chain fatty acids are preferentially hydrolyzed by pancreatic lipase. This evidence was obtained by hydrolysis of milk fat samples before and after randomization. The fatty acid composition of partial glycerides indicated the rate at which various fatty acids were split off.

It is not yet known whether this kind of specificity arises from a better orientation of the short chain fatty acids when the complex lipase-substrate is formed or from a quicker transfer of these fatty acids after the complex formation.

An attempt was made to explain the pathway by which pancreatic lipase hydrolyzes milk fat glycerides.

The general conclusions reached by investigators using the pancreatic lipase technique is that most natural fats do not have a random structure. This investigation provided evidence that the fatty acid distribution of milk fat is specific.

The classical definition of milk fat as being

essentially a mixture of mixed triglycerides should be revised for the presence of characteristic amounts of diglycerides and monoglycerides. The relatively high content of short chain fatty acids which were found in the natural diglycerides of milk fat might require revisions of the concept of triglyceride synthesis which has been proposed by Patton and McCarthy.

BIBLIOGRAPHY

1. Artom, C., and Zummo, C. (1937).
Digestione Pancreatica degli Esteri Glicerici a, a-Dicapril-B-palmitica e a, a-Dipalmito-B-caprilico.
Enzymologia, 3:234.

2. Ast, H.J. and Vander Wal, R.J. (1961).
The Structural Components of Milk Triglycerides.
J.Am. Oil Chemists' Soc., 38:67.

3. Balls, A.K. and Matlack, M.B. (1938).
Mode of Action of Pancreatic Lipase
J. Biol.Chem., 123:679.

4. Balls, A.K., Matlack, N.B. and Tucker, I.W. (1937).
The Hydrolysis of Glycerides by Crude Pancreatic Lipase.
J. Biol.Chem., 122:125.

5. Barron, E.J. and Hanahan, D.J. (1958).
Observations on the Silicic Acid Chromatography of the Neutral Lipides of Rat Liver, Beef Liver and Yeast.
J. Biol.Chem., 231:493.

6. Bernhard, K., Wagner, H. und Ritzel, G. (1952).
Versuche zur Quantitativen Erfassung der bei der Resorption von Neutralfett Eintretenden Spaltung.
Helv. Chim.Acta, 35:1404.

7. Borgström, B. (1956).
Biochemical Problems of Lipids. p.179
G.Popjak and E.Le Breton, ed.
London Butterworth Scientific Publications.

8. Borgström, B. (1954).
On the Mechanism of Pancreatic Lipolysis of Glycerides.
Biochim. et Biophys. Acta, 13:491.

9. Borgström, B. (1952).
On the Action of Pancreatic Lipase on Triglycerides in Vivo and in Vitro.
Acta Physiol. Scand., 25:328.

10. Borgström, B. (1955).
Randomization of Glyceride Fatty Acid during Absorption from the Small Intestine of the Rat.
J.Biol.Chem., 214:671.

11. Borgström, B. (1953).
On the Mechanism of the Hydrolysis of Glycerides by Pancreatic Lipase.
Acta Chem. Scand., 7:557.

12. Borgström, B. (1952).
Investigation on Lipid Separation Methods, Separation of Cholesterol Esters, Glycerides and Free Fatty Acids.
Acta Physiol. Scand. 25:111.

13. Carrol, K.K (1961).
Separation of Lipid Classes by Chromatography on Florisil.
J.Lipid Res., 2:135.

14. Clément, G., Clément, J. et Bézard, J. (1962).
Action de la Lipase Pancréatique Humaine sur des Triglycérides Mixtes, Symétriques de Synthèse Comportant des Acides Gras Longs et de l'Acide Butyrique.
Arch. Sci. Physiol., 16:213.

15. Clément, G., Clément, J., Bézard, J., Di Costanzo, G. et Paris, R. (1962). Hydrolyse des Triglycérides de Beurre par la Lipase Pancréatique. Localisation de l'Acide Butyrique. Arch.Sci.Physiol., 16:237.

16. Coleman, M.H. (1963). The Pancreatic Hydrolysis of Natural Fats. III. The Influence of the Extent of Hydrolysis on Monoglyceride Composition. J.Am.Oil Chemists' Soc., 40:568.

17. Coleman, M.H. (1961). Further Studies on the Pancreatic Hydrolysis of some Natural Fats. J.Am.Oil Chemists' Soc., 38:685.

18. Coleman, M.H. and Fulton, W.C. (1961). The Enzymes of Lipid Metabolism. p.127. P.Desnuelle, ed. Pergamon Press, Oxford.

19. Constantin, M.J. Pasero, L. et Desnuelle, P. (1960). Quelques Remarques Complémentaires sur l'Hydrolyse des Triglycérides par la Lipase Pancréatique. Biochim.et Biophys.Acta, 43:103.

20. deMan, J.M. (1964). In press, J.Dairy Sci.

21. deMan, J.M. (1961). Gas Chromatography of the Short-Chain Fatty Acids of Milk Fats. Milchwissenschaft, 16:245.

22. deMan, J.M. (1961).
Physical Properties of Milk Fat.
I. Influence of Chemical Modification.
J.Dairy Res., 28:81.

23. deMan, J.M. and Bowland, J.P. (1963).
Fatty Acid Composition of Sow's
Colostrum, Milk and Body Fat as
Determined by Gas-Liquid-Chromatography.
J.Dairy Res., 30:339.

24. Desnuelle, P. (1951).
Hydrolyse Enzymatique des Triglycérides.
Bull.Soc.Chim.biol., 33:909.

25. Desnuelle, P., Naudet, M. et Constantin, M.J. (1950).
Action des Sels de Calcium sur l'Hydrolyse
des Triglycérides par la Pancréatine et le
Suc Pancréatique.
Biochim.et Biophys.Acta, 5:561.

26. Desnuelle, P., Naudet, M. et Constantin, M.J. (1951).
Sur un Nouveau Type de Lipolyse "In Vitro",
une Lipolyse "Génératrice de Glycérol".
Biochim.et Biophys.Acta, 7:251.

27. Desnuelle, P., Naudet, M. et Rouzier, J. (1948).
Etude Quantitative de la Formation de
Glycérides Partiels au cours de l'Hydrolyse
Fermentaire des Triglycérides.
Biochim.et Biophys.Acta, 2:561.

28. Desnuelle, P. and Savary, P. (1963).
Specificities of Lipases.
J.Lipid Res., 4:369.

29. Desnuelle, P. et Savary, P. (1959).
Sur la Structure Glycéridique de quelques
Corps Gras Naturels.
Fette-Seifen-Anstrichm., 61:871.

30. Doerschuk, A.P. and Daubert, B.F. (1948).
Low-Temperature Solvent Fractionation of Corn Oil and Calculation of its Glyceride Structure.
J.Am.Oil Chemists' Soc., 25:425.

31. Entressangles, B., Pasiro, L., Savary, P., Sarda, L. et Desnuelle, P. (1961).
Influence de la Nature des Chaînes sur la Vitesse de leur Hydrolyse par la Lipase Pancréatique.
Bull.Soc.Chim.Biol., 43:581.

32. Favarger, P., Collet, R.A. et Cherbuliez, E. (1951).
Etude de la Résorption Intestinale des Graisses a l'aide de Deutéro-Glycérol et d'Acides Gras Marqués.
Helv.Chim.Acta, 34:1641.

33. Frazer, A.C. and Sammons, H.G. (1945).
The Formation of Mono- and Di-glycerides during the Hydrolysis of Triglycerides by Pancreatic Lipase.
Biochem.J., 39:122.

34. Gunstone, F.D. (ed.) (1958).
An Introduction to the Chemistry of Fats and Fatty Acids. p.72
London.

35. Harris, R.S., Chamberlain, J.W. and Benedict, J.H. (1955).
Digestion of Neutral Fats by Human Subjects.
J.Clin.Invest., 34:685.

36. Herb, S.F., Magidman, P., Luddy, F.E. and Riemenschneider, R.W. (1962).
Fatty Acids of Cows' Milk. B. Composition by Gas-Liquid Chromatography Aided by other Methods.
J.Am.Oil Chemists' Soc., 39:142.

37. Hernandez, H.H. and Chaikoff, I.L. (1957).
Purification and Properties of Pancreatic Cholesterol Esterase.
J Biol.Chem., 228:447.

38. Hilditch, T.P. (1949).
A Contribution to the Discussion of the Glyceride Structure of Stearic-Rich Animal Depot Fats.
J.Am.Oil Chemists' Soc., 26:41.

39. Hilditch, T.P. (1956).
The Chemical Constitution of Natural Fats.
John Wiley & Sons Inc., New York.

40. Hirsch, J. and Ahrens, E.H. Jr. (1959).
The Separation of Complex Lipide Mixtures by the Use of Silicic Acid Chromatography.
J.Biol.Chem., 233:311.

41. Hofstee, B.H.J. (1952).
Specificity of Esterases. I. Identification of Two Pancreatic Aliesterases.
J.Biol.Chem., 199:357.

42. Hofstee, B.H.J. (1952).
Specificity of Esterases. II. Behaviour of Pancreatic Esterases I and II towards a Homologous Series of n-Fatty Acid Esters.
J.Biol.Chem., 199:365.

43. Jack, E.L. (1960).
The Fatty Acids and Glycerides of Cow's Milk Fat.
J.Agr.Food Chem., 8:377.

44. Jack, E.L., Freeman, C.P., Smith, L.M. and Mickle, J.B. (1963).
Pancreatic Lipase Hydrolysis of Cow Milk Fat.
J.Dairy Sci., 46:284.

45. Jack, E.L. and Smith, L.M. (1956).
Chemistry of Milk Fat: A Review.
J.Dairy Sci., 39:1.

46. Jenness, R. and Patton, S. (1959).
Principles of Dairy Chemistry.
John Wiley and Sons Inc., New York.

47. Jensen, R.G. and Gander, G.W. (1960).
Fatty Acid Composition of the Monoglycerides from Lipolyzed Milk Fat.
J.Dairy Sci., 43:1758.

48. Jensen, R.G., Gander, G.W. and Duthrie, A.H. (1959).
Total Monoglyceride Content of some Dairy Products.
J. Dairy Sci., 42:1913.

49. Jensen, R.G. and Morgan, M.E. (1959).
Estimation of the Monoglyceride Content of Milk.
J.Dairy Sci., 42:232.

50. Jensen, R.G., Sampugna, J. and Gander, G.W. (1961).
Fatty Acid Composition of the Diglycerides from Lipolyzed Milk Fat.
J. Dairy Sci., 44:1983.

51. Kartha, A.R.S. (1953).
The Glyceride Structure of Natural Fats.
I. A Technique for the Quantitative Determination of Glyceride Types in Natural Fats.
J.Am.Oil Chemists' Soc., 30:280.

52. Kartha, A.R.S. (1953).
The Glyceride Structure of Natural Fats.
II. The Rule of Glyceride Type Distribution of Natural Fats.
J.Am.Oil Chemists' Soc., 30:326.

53. Kartha, A.R.S. (1954).
The Glyceride Structure of Natural Fats. III. Factors Governing the Content of Fully Saturated Glycerides.
J.Am.Oil Chemists' Soc., 31:85.

54. Kaufmann, H.P. und Mankel, G. (1963).
Anwendung der Gas-Chromatographie auf dem Fettgebiet III:Einfluss der Herstellungsverfahren der Fettsäure - methylester auf das quantitative Ergebnis.
Fette-Seifen-Anstrichm., 65:179.

55. Keller, P.J., Cohen, E. and Neurath, H. (1958).
The Proteins of Bovine Pancreatic Juice.
J.Biol.Chem., 233:344.

56. Kumar, S. and Lalka, K. (1960).
Butyric Acid Incorporation in Bovine Milk Fat Synthesis.
Fed. Proc., 19 (1) Pt.1:228.

57. Kumar, S., Pynadath, T.L. and Lalka, K. (1963).
Location of Butyric Acid in Milk Triglycerides.
Biochim.et Biophys.Acta, 42:373.

58. Lambert, Marguerite, and Neish, A.C. (1950).
Rapid Method for Estimation of Glycerol in Fermentation Solutions.
Canadian J.Res., 28B:83.

59. Longenecker, H.E. (1941).
Composition and Structural Characteristics of Glycerides in Relation to Classification and Environment.
Chem.Rev., 29:201.

60. MacFayden, D.A. (1945).
Estimation of Formaldehyde in Biological Mixtures.
J.Biol.Chem., 158:107.

61. Malins, D.C. and Mangold, H.K. (1960).
Analysis of Complex Lipid Mixtures by Thin-Layer Chromatography and Complementary Methods.
J.Am.Oil Chemists' Soc., 37:576.

62. Mangold, H.K. and Malins, D.C. (1960).
Fractionation of Fats, Oils and Waxes on Thin Layers of Silicic Acid.
J.Am.Oil Chemists' Soc., 37:383.

63. Marchis-Mouren, G., Charles, M. et Ben Abdeljlil, A., Desnuelle, P. (1961).
Sur l'Equipment en Enzymes du Suc Pancréatique de Porc et de Chien.
Biochim.et Biophys.Acta, 50:186.

64. Marchis-Mouren, G., Sarda, L. et Desnuelle, P. (1960).
Purification de la Lipase à partir du Suc Pancréatique de Porc.
Biochim.et Biophys.Acta, 41:358.

65. Marchis-Mouren, G., Sarda, L. and Desnuelle, P. (1959).
Purification of Hog Pancreatic Lipase.
Arch.Biochem.Biophys., 83:309.

66. Martin, J B. (1953).
The Equilibrium between Symmetrical and Unsymmetrical Monoglycerides and Determination of Total Monoglycerides.
J.Am.Chem.Soc., 75:5483.

67. Mattson, F.H. and Beck, L.W. (1955).
The Digestion in Vitro of Triglycerides by Pancreatic Lipase.
J.Biol.Chem., 214:115.

68. Mattson, F.H. and Beck, L.W. (1956).
The Specificity of Pancreatic Lipase for the Primary Hydroxyl Groups of Glycerides.
J.Biol.Chem., 219:735.

69. Mattson, F.H., Benedict, J.H. and Beck, L.W. (1954).
Composition of Intestinal Lumen Lipides Following the Feeding of Triglycerides, Partial Glycerides or Free Fatty Acids.
J.Nutr., 52:575.

70. Mattson, F.H., Benedict, J.H., Martin, J.B. and Beck, L.W. (1952).
Intermediates Formed during the Digestion of Triglycerides.
J.Nutr., 48:335.

71. Mattson, F.H. and Lutton, E.S. (1958).
The Specific Distribution of Fatty Acids in the Glycerides of Animal and Vegetable Fats.
J.Biol.Chem., 233:868.

72. Mattson, F.H. and Volpenhein, R.A. (1962).
Synthesis and Properties of Glycerides.
J.Lipid Res., 3:281.

73. Mattson, F.H. and Volpenhein, R.A. (1961).
The Specific Distribution of Fatty Acids in the Glycerides of Vegetable Fats.
J.Biol.Chem., 236:1891.

74. Mattson, F.H. and Volpenhein, R.A. (1961). The Use of Pancreatic Lipase for Determining the Distribution of Fatty Acids in Partial and Complete Glycerides. J.Lipid Res., 2:58.

75. McCarthy, R.D., Patton, S. and Evans, Laura, (1960). Structure and Synthesis of Milk Fat. II. Fatty Acid Distribution in the Triglycerides of Milk and other Animal Fats. J.Dairy Sci., 43:1196.

76. Mehlenbacher, V.C. (1960). The Analysis of Fats and Oils. Garrard Press, Champaign, Illinois.

77. Mehlenbacher, V.C. and Hopper, T.H. (eds.) (1957). Official and Tentative Methods of the American Oil Chemists' Society. American Oil Chemists' Society, Chicago.

78. Mickle, J.B., Von Gunten, R.L. and Morrisson, R.D. (1963). Rearrangement of Milk Fat as a Means for Adjusting Hardness of Butterlike Products. J.Dairy Sci., 46:1357.

79. Norris, F.A. and Mattil, K.F. (1947). A New Approach to the Glyceride Structure of Natural Fats. J.Am.Oil Chemists' Soc., 24:274.

80. Nutritional Biochemicals Corporation (1963). Technical Specifications Cleveland 28, Ohio.

81. Patton, S. (1960). Gas Chromatographic Analysis of Milk Fat. J.Dairy Sci., 43:1350.

82. Patton, S., Evans, Laura, and McCarthy, R.D. (1960).
The Action of Pancreatic Lipase on Milk Fat.
J.Dairy Sci., 43:95.

83. Patton, S. and McCarthy, R.D. (1963).
Structure and Synthesis of Milk Fat.
V. A Postulated Sequence of Events from Analyses of Mammary Tissue Lipids.
J.Dairy Sci., 46: 916.

84. Peterson, E.A. and Sober, H.A. (1956).
Chromatography of Proteins. I.
Cellulose Ion-Exchange Adsorbents.
J.Am.Chem.Soc., 78:751.

85. Privett, O.S., Blank, M.L. and Lundberg, M.O. (1961).
Determination of Mono-, Di-, and Triglycerides by Molecular-Distillation and Thin-Layer Chromatography.
J.Am.Oil Chemists' Soc., 38:312.

86. Quimby, O.T., Wille, R.L. and Lutton, E.S. (1953).
On the Glyceride Composition of Animal Fats.
J.Am.Oil Chemists' Soc., 30:186.

87. Reiser, R., Bryson, M.J., Can, M.J. and Kuicken, K.A. (1952).
The Intestinal Absorption of Triglycerides.
J.Biol.Chem., 194:131.

88. Reiser, R. and Reddy, H.G.R. (1959).
The Glyceride Structure of Swine Depot Fat.
J.Am.Oil Chemists' Soc., 36:97.

89. Sarda, L. et Desnuelle, P. (1958).
Action de la Lipase Pancréatique sur les Esters en Emulsion.
Biochim.et Biophys.Acta, 30:513.

90. Savary, P. et Desnuelle , P. (1956).
Sur quelques Eléments de Spécificité pendant l'Hydrolyse Enzymatique des Triglycérides.
Biochim.et Biophys.Acta, 21:349.

91. Savary, P. et Desnuelle, P. (1955).
Chimie Biologique. - Etude Chromatographique de l'Action de la Lipase Pancréatique sur des Triglycérides Mixtes.
Compt.rend. 240:2571.

92. Savary, P., Flanzy, J. et Desnuelle, P. (1957).
Emploi de la Lipase Pancréatique pour l'Etude de la Structure des Corps Gras Naturels.
Biochim.et Biophys.Acta, 24:414.

93. Schönheyder, F. and Volquartz, K. (1952).
Studies on the Lipolytic Enzyme Action. III. Hydrolysis of Tripropionyl Glycerol.
Biochim.et Biophys.Acta, 8:407.

94. Schönheyder, F. and Volquartz, K. (1954).
Studies on the Lipolytic Enzyme Action. VI. Hydrolysis of Trilauryl Glycerol by Pancreatic Lipase.
Biochim.et Biophys.Acta, 15:288.

95. Sims, R.P.A. and Larose, J.A.F. (1962).
The use of Iodine Vapor as a General Detecting Agent in the Thin Layer Chromatography of Lipids.
J.Am.Oil Chemists' Soc., 39:232.

96. Tattrie, N.H., Bailey, R.A. and Kates, M. (1956).
The Action of Pancreatic Lipase on Stereoisomeric Triglycerides.
Arch.Biochem.Biophys., 78:319.

97. Vander Wal, R.J. (1960).
The Glyceride Structure of Fats and Oils.
J.Am.Oil Chemists' Soc., 37:595.

98. Vander Wal, R.J. (1960).
Calculation of the Distribution of the Saturated and Unsaturated Acyl Groups in Fats, from Pancreatic Lipase Hydrolysis Data.
J.Am.Oil Chemists'Soc., 37:18.

99. Weber, H.H.R. and King, C.G. (1935).
Specificity and Inhibition Characteristics of Liver Esterase and Pancreas Esterase.
J.Biol.Chem., 108:131.

100. Wills, E.D. (1954).
The Effect of Anionic Detergents and some Related Compounds on Enzymes.
Biochem.J., 57:109.

101. Wills, E.D. (1958).
The Purification of Pancreatic Lipase Using Zone Electrophoresis on Plastic Sponge.
Biochem.J., 69:17P.

102. Wills, E.D. (1961).
The Enzymes of Lipid Metobolism.
p.13. P.Desnuelle, ed.
Pergamon Press, New York.

103. Youngs, C.G. (1959).
Glyceride Structure of Fats.
J.Am.Oil Chemists' Soc., 36:664.

B29819